University of Alberta

Biological Significance of Signal Transducer and Activator of Transcription-3 in the Pathogenesis of Cancer

by

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I dedicate this thesis to my dad, who I will always love and will never forget.

ABSTRACT

Signal Transducers and Activators of Transcription (STAT) comprise a unique family of latent transcription factors that transmit the interactions of cytokines and growth factors with their cognate receptors to participate in various physiological functions, including cell growth, differentiation, and survival. Among the STAT family, increasing evidence demonstrates that aberrant activation of STAT3 play an important role in the pathogenesis of a large portion of neoplasms, by promoting cell cycle progression and survival, preventing apoptosis, and impairing tumor immune surveillance. However, the mechanisms responsible for the tumorigenic activity of STAT3 have only partially been elucidated. Further research to identify novel targets in the STAT3 signaling pathway may prove efficacious for cancer treatment.

The first objective of this thesis examined the novel link between STAT3 and TIMP1, a glycoprotein known to decrease cell migration and invasion, in breast cancer. STAT3 activation was shown to up-regulate TIMP1 expression and modulate the invasive abilities of breast cancer cell lines. Moreover, it was confirmed that STAT3 is highly activated in human breast cancer and correlates with a decrease in lymphatic and vascular invasion.

The second objective of this thesis examined the aberrant activation of STAT3 by two autocrine stimulatory pathways. First, aberrant expression of IL-22R1, a receptor that is absent in normal immune cells, was identified in ALK^+ALCL. NPM-ALK, the characteristic fusion gene oncoprotein in ALK^+ALCL, was linked to the aberrant

expression of IL-22R1. Second, an autocrine stimulatory pathway for IL-21 and its corresponding receptor IL-21R was also identified in ALK^{+}ALCL and found to contribute to the cell growth potential in these cell lines.

The third objective of this thesis examined the novel link between STAT3 and SALL4, a newly denoted oncogene that is essential in self-renewal of embryonic stem cells. STAT3 was demonstrated to directly up-regulate *SALL4* gene transcription by binding to specific STAT3-binding sites on the *SALL4* gene promoter and appears to contribute to the aberrant expression of SALL4 in breast cancer.

Overall, the identification of these novel upstream stimulators and downstream targets of STAT3 furthered our current understanding of STAT3 signaling and provides a framework for development of novel anti-cancer drugs.

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TABLE OF CONTENTS

		Page
Chapter 1 ♦ General Introduction		1
1.1.	Introduction	2
1.2.	STAT family of transcription factors (6 Domains)	3
	1.2.1. Domains of STAT proteins	5
	1.2.2. Isoforms of STAT proteins	11
1.3.	Regulations of STAT proteins	11
	1.3.1. Phosphorylation	12
	1.3.2. Arginine methylation, lysine acetylation, and ISGylation	14
1.4.	Nuclear trafficking of STAT3 protein	15
	1.4.1. Nuclear import	17
	1.4.2. Nuclear export	18
1.5.	Activation mechanisms of STAT3 protein	19
	1.5.1. Cytokine	21
	1.5.2. Growth factors	25
	1.5.3. Janus Kinase family	25
	1.5.4. Non-receptor tyrosine kinases	28
1.6.	Negative regulation of STAT3	28
	1.6.1. Cytoplasmic tyrosine phosphatases	28
	1.6.2. Nuclear regulators	29
	1.6.3. Suppressor of cytokine signaling	30

1.7. Biological functions of STAT3 32

1.7.1. Requirement of STAT3 for embryonic development 32

1.7.2. Conditional knockout of STAT3 35

1.7.3. Embryonic stem cell 38

1.8. Role of STAT3 in oncogenesis 39

1.9. Target genes regulated by STAT3 43

1.10. STAT3 activation in human cancer 48

1.10.1. Breast cancer 48

1.10.2. Anaplastic lymphoma kinase-positive Anaplastic large cell lymphoma 49

1.11. Cancer therapeutics for STAT3 50

1.12. Thesis overview and objectives 52

1.13. References 54

Chapter 2 ♦ STAT3 upregulates TIMP1 expression and decreases invasiveness of breast cancer 73

2.1. Introduction 74

2.2. Materials and Methods 76

2.2.1. Construction of Tet-off STAT3C 76

2.2.2. Cell lines and Culture 77

2.2.3. Transfection 78

2.2.4. Western Blot Analysis and Antibodies 78

2.2.5 Immunofluorescence Staining and Confocal Microscopy ... 79

2.2.6. Matrigel Invasion Assay 80

2.2.7 ELISA Assay for TIMP1 81

2.2.8 Immunohistochemistry 81

2.2.9 Patient and Tumor Specimens 82

2.2.10 Statistical Analysis 83

2.3. Results 83

2.3.1. Characterization of MCF-7 transfected with tTA-TRE-STAT3C 83

2.3.2. STAT3 transgene correlated with TIMP1 expression 89

2.3.3. Modulation of invasiveness by TIMP1 and STAT3 91

2.3.4. Correlation between STAT3 activation and TIMP1 expression, lymphatic, and vascular invasion in breast cancer primary tumors 94

2.3.5. Correlation between STAT3 activation and TIMP1 expression and other clinical parameters in breast cancer patients 97

2.4. Discussion 97

2.5. References 106

Chapter 3 ♦ Aberrant expression of IL-22 receptor 1 (IL-22R1) and autocrine IL-22 stimulation contribute to tumorigenicity in ALK-positive anaplastic large cell lymphoma 112

3.1. Introduction 113

3.2. Materials and Methods 116

3.2.1. Cell lines and Culture 116

3.2.2. Treatment of Cells 116

3.2.3. Western Blot Analysis and Antibodies 117

3.2.4. Immunofluorescence Staining and Confocal Microscropy ... 118

3.2.5. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) ... 119

3.2.6. IL-22R1 siRNA ... 122

3.2.7. Gene Transfection of NPM-ALK ... 122

3.2.8. ELISA Assay for IL-22 ... 123

3.2.9. Cell Growth Assay ... 123

3.2.10. Colony Formation in Soft Agar ... 124

3.2.11. ALK$^+$ALCL Tumors and Immunohistochemistry ... 124

3.2.12. Statistical Analysis ... 125

3.3. Results ... 126

3.3.1. Expression of IL-22R1, IL10R2, and IL-22 in ALK$^+$ALCL cell lines and tumors ... 126

3.3.2. IL-22 modulated cell growth in ALK$^+$ALCL ... 134

3.3.3. IL-22 modulated tumorigenicity assessed by colony formation in soft agar ... 140

3.3.4. IL-22 modulated STAT3 and MAPK signaling ... 143

3.3.5. NPM-ALK induced IL-22R1 expression ... 149

3.4. Discussion ... 152

3.5. References ... 159

Chapter 4 ♦ IL-21 contributes to JAK3/STAT3 activation and promotes cell growth in ALK-positive anaplastic large cell lymphoma ... 165

4.1. Introduction ... 166

4.2. Materials and Methods ... 169

4.2.1. Cell lines and Culture 169

4.2.2. Treatment of Cells 169

4.2.3. Western Blot Analysis and Antibodies 169

4.2.4. Immunofluorescence Staining and Confocal Microscropy .. 170

4.2.5. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) 171

4.2.6. Cell Growth Assay 174

4.2.7. siRNA Transfection 174

4.2.8. MTS Assay 174

4.2.9. ALK^+ALCL Tumors and Immunohistochemistry 175

4.2.10. Statistical Analysis 176

4.3. Results 176

4.3.1. Expression of IL-21 and IL-21R in ALK^+ALCL cell lines and tumors 176

4.3.2. IL-21 modulated JAK3/STAT3 signaling 184

4.3.3. IL-21 treatment increased cell growth of ALK^+ALCL cell lines 184

4.4. Discussion 190

4.5. References 195

Chapter 5 ♦ SALL4 is directly activated by STAT3 in cancer 200

5.1. Introduction 201

5.2. Materials and Methods 203

5.2.1. Construction of Tet-off STAT3C 203

5.2.2. *SALL4* Promoter Construct 204

5.2.3. Cell lines and Culture 204

5.2.4. Transfection 205

5.2.5. Western Blot Analysis and Antibodies 205

5.2.6. Nuclear Extraction 206

5.2.7. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) 207

5.2.8. Luciferase Assay 208

5.2.9. Chromatin Immunoprecipitation (ChIP) assay 209

5.2.10. Site Specific Mutagenesis 210

5.2.11. STAT3DN Adenoviral Vector 211

5.2.12. Generation of SALL4 siRNA Retrovirus 212

5.2.13. MTS Assay 212

5.3. Results 213

5.3.1. SALL4 gene promoter contains the STAT3 consensus sequence 213

5.3.2. STAT3 regulates *SALL4* gene expression 216

5.3.3. STAT3 binds to *SALL4* Promoter 221

5.3.4. Detection of SALL4 in human cell lines 225

5.3.5. Effects of endogenous SALL4 on human cancer cell lines 230

5.4. Discussion 233

5.5. References 238

Chapter 6 ♦ Summary and Future Directions 244

6.1. Thesis overview 245

6.2. STAT3 activation by upstream regulators 246

6.3. STAT3-dependent activation of target genes 248

6.4. Therapeutic implications of STAT3 251

6.5. Closing remarks 253

6.6. References 255

LIST OF TABLES

	Page
Table 1.1. STAT protein properties	4
Table 1.2. Activation of STAT proteins by various ligands	22
Table 1.3. STAT Knockout studies	34
Table 1.4. STAT3 conditional knockout study	37
Table 1.5. STAT activation in human tumors and cell lines	42
Table 1.6. STAT3-regulated target genes	47
Table 2.1. Correlation between STAT3 activation and TIMP1 expression, Lymphatic invasion and vascular invasion	96
Table 3.1. Primer Sequences and Product Size	121
Table 4.1. Primer Sequence and PCR conditions	173
Table 5.1. Potential STAT3-binding sites in human *SALL4* promoter	214
Table 5.2. Potential STAT3-binding sites in mouse *SALL4* promoter	215

LIST OF FIGURES

Page

Figure 1.1. Structure of STAT protein 9

Figure 1.2. STAT domain functions 10

Figure 1.3. Schematic illustration of STAT3 protein import/export cycle 16

Figure 1.4. Activation mechanisms of STAT3 20

Figure 1.5. Phosphorylation of JAK by gp130 24

Figure 1.6. JAK combinations utilized by cytokine receptors 27

Figure 2.1. Characterization of MCF-7 transfected with tTA-TRE-STAT3C 85

Figure 2.2. Characterization of MDA-MB-436 transfected with tTA-TRE-STAT3C 87

Figure 2.3. Nuclear localization of STAT3C 88

Figure 2.4. STAT3C correlated with TIMP1 levels 90

Figure 2.5. STAT3 and TIMP1 levels inversely correlated with invasion 92

Figure 2.6. TIMP1 directly modulated cell invasion 93

Figure 2.7. pSTAT3 and TIMP1 expression in primary breast tumors 95

Figure 3.1. mRNA expression of IL-22 receptors in ALK^+ALCL 127

Figure 3.2. Protein expression of IL-22 receptors in ALK^+ALCL 128

Figure 3.3. IL-22R1 expression in ALK^+ALCL cell lines 129

Figure 3.4. Autocrine expression of IL-22 in ALK^+ALCL cell lines 131

Figure 3.5. IL-22 and IL-22R1 expression in ALK^+ALCL tumors 132

Figure 3.6. Double staining of ALK^+ALCL tumor with IL-22 and IL-22R1 133

Figure 3.7. IL-22 modulated cell proliferation in ALK^+ALCL cell lines 136

Figure 3.8. IL-22 modulated cell proliferation in ALK^+ALCL cell lines 137

Figure 3.9. IL-22 modulated cell proliferation in ALK$^+$ALCL cell lines using MTS assay 139

Figure 3.10. IL-22 modulated ALK$^+$ALCL colony formation 141

Figure 3.11. Effects of IL-22 on STAT3 signaling pathway 145

Figure 3.12. Effects of IL-22 on STAT3 and MAPK signaling pathway 146

Figure 3.13. IL-22R1 directly up-regulated STAT3 activation 148

Figure 3.14. Induction of IL-22R1 expression in NPM-ALK activated cell line ... 150

Figure 3.15. Induction of STAT3 activation in NPM-ALK activated cell line 151

Figure 3.16. A hypothetical model of the IL-22 autocrine pathway in ALK$^+$ALCL 156

Figure 4.1. mRNA expression of IL-21 and IL-21R in ALK$^+$ALCL cell lines 177

Figure 4.2. Protein expression of IL-21R in ALK$^+$ALCL 180

Figure 4.3. IL-21R expression in ALK$^+$ALCL cell lines 181

Figure 4.4. mRNA expression of IL-21 and IL-21R in ALK$^+$ALCL tumor 182

Figure 4.5. IL-21 and IL-21R expression in ALK$^+$ALCL tumors 183

Figure 4.6. Effects of IL-21 on STAT3, JAK3, STAT1 signaling 186

Figure 4.7. IL-21 modulated cell proliferation in ALK$^+$ALCL cell lines 187

Figure 4.8. IL-21 directly up-regulated STAT3 and cell growth 188

Figure 5.1. SALL4 expression in STAT3C $^{tet\text{-}off}$ system 218

Figure 5.2. mRNA SALL4 expression in STAT3C $^{tet\text{-}off}$ system 219

Figure 5.3. SALL4 transcriptional activation is STAT3 dependent 220

Figure 5.4. STAT3 binds to the human *SALL4* promoter 222

Figure 5.5. Transcriptional activation of *SALL4* promoter with altered STAT3-binding sites compared to wild type 224

Figure 5.6. Expression of SALL4 in breast cancer 227

Figure 5.7. Nuclear SALL4 correlated with pSTAT3 in breast cancer 228

Figure 5.8. STAT3 up-regulated SALL4 expression 229

Figure 5.9. SALL4 promote cell growth in breast cancer 231

LIST OF ABBREVIATIONS

AEBSF – 4-(2-aminoethyl)-benzenesulfonyl fluoride

ALK – Anaplastic lymphoma kinase

ALK^+ALCL – Anaplastic lymphoma kinase positive anaplastic large cell lymphoma

AML – Acute myeloid leukemia

βB – β-strand

β-GAL – β-galactosidase

Bcl-2 – B-cell lymphoma-2

BCR-Abl – Breakpoint cluster region-Abl

bFGF – Basic fibroblast growth factor

Blg – β-lactoglobulin

BRCA1 – Breast cancer susceptibility gene 1

C-terminal – Carboxy-terminal

CBP/p300 – Camp-responsive element binding protein

CDK – Cyclin-dependent kinase

ChIP – Chromatin Immunoprecipitation

CIS – Cytokine inducible SH2 domain-containing

CNTF – Ciliary neurotrophic factor

CNTFR – CNTF receptor

CRM1 – Chromosome region maintenance 1

CSF-1 – Colony stimulating factor 1

DMEM – Dulbecco's Modified Eagle's medium

DRAQ5 – deep red fluorescing anthraquinone Nr.5

DTT – Dithiothreitol

EBV – Epstein-Barr virus

EDTA – Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

EGFR – EGF receptor

Epo – Erythropoietin

ERK1/2 – Extracellular-signal-regulated kinase 1/2

ES – Embryonic stem

ESP – Extracellular signaling proteins

FBS – Fetal bovine serum

G-CSF – Granulocyte colony stimulating factor

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GAS – Gamma interferon gene response

GFP – Green fluorescent protein

GM-CSF – Granulocyte/macrophage colony stimulating factor

HCL – Hydrochloride

HGF – Hepatocyte growth factor

HIF-1α – Hypoxia-inducible factor-1α

HPC – Hematopoietic progenitor cells

HSC – Hematopoietic stem cells

HTLV-1 – Human T-lymphotropic virus-1

ICM – Inner cell mass

IGFBP5 – Insulin-like growth factor binding protein 5

IL – Interleukin

INF – Interferon

ISFG3 – IFN-stimulated gene factor 3 complex

JAK – Janus Kinase

JNK – c-Jun NH_2-terminal kinases

K5 – Keratin 5

Lck – Lymphocyte specific protein tyrosine kinase

LEF1 – Lymphoid enhancer binding factor 1

LIF – Leukemia inhibitory factor

LIFR – LIF receptor

LPMSP – Leu-Pro-Met-Ser-Pro

MAPK – Mitogen-activated protein kinase

Mcl-1 – Myeloid cell leukemia-1

$MgCO_3$ - Magnesium carbonate

MgSO4 – Magnesium sulphate

MHC – Major histocompatibility complex

Mlys – Macrophage lysozyme

MMP – Matrix metalloproteinases

MUC1 – Mucin 1

N-terminal – Amino-terminal

NESs – Nuclear –export signals

NF-κB – Nuclear factor-kappa B

NLSs – Nuclear-localization signals

NPCs – Nuclear pore complexes

NPM – Nucleophosmin

NPM-ALK – Nucleophosmin-anaplastic lymphoma kinase

ONPG – Ortho-Nitrophenyl- β-galactoside

OSM – Oncostatin M

PBS – Phosphate-Buffer Saline

PCR – Polymerase chain reaction

PDGF – Platelet derived growth factor

PI-3K – Phosphatidylinositol 3-kinase

PIAS – Protein inhibitor of activated STAT

PMSP – Pro-Met-Ser-Pro

PRMT1 – Protein arginine methytransferase 1

PSP – Pro-Ser-Pro motif

PTPs – Protein tyrosine phosphatases

PTP1B – Protein-tyrosine-phosphatase-1B

pTyr – Phosphotyrosine

RHS – Rapid microwave histoprocessor

RT – Reverse transcription

RT-PCR – Reverse transcriptase-polymerase chain reaction

SCID – Severe combined immunodeficient

SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM – Standard error of mean

SH2 – Src homology 2

SHP1 – SH2-containing phosphatase-1

SOCS – Suppressor of cytokine signaling

STAT – Signal transducers and activators of transcription

STAT3C – Constitutively active STAT3

SUMO – Small-ubiquitin like modifier

TAD – Transcriptional activation domain

Tc-PTP – T-cell protein tyrosine phosphatase

TGF – Tumor growth factor

TIMP – Tissue inhibitor of metalloproteinases

TNF – Tumor necrosis factor

TNFR – TNF receptor

TRE – Tetracycline response element

TSB – Tris-Buffer Saline

tTa – Tetracycline-controlled transactivator

VEGF – Vascular endothelial growth factor

WSxWS – Tryptophan-serine-X-tryptophan-serine

Chapter 1 ♦

General Introduction

1.1. Introduction

Extensive research has led to the important finding that ligand-receptor interaction at the cell surface, particularly receptors that bind extracellular signaling proteins (ESP), results in the transcriptional activation of latent genes. ESPs are also referred to as cytokines including, interferon (INF)-α, INF-β, INF-γ, and interleukins (IL), or growth factors such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF) (Levy and Darnell, 1990). In the early 1990s, studies examining the mediation of transcriptional activation by INFs led to the discovery of a family of related proteins defined as Signal Transducers and Activators of Transcription (STAT) (Darnell, 1997; Darnell et al., 1994). STAT proteins are a group of cytoplasmic transcriptional factors found to mediate responses to a large number of ESPs and are implicated in a wide variety of biological responses, including development, differentiation, cell proliferation and cell survival (Brierley and Fish, 2005; Darnell, 1997). Of the STAT family, STAT3 was initially characterized as a DNA-binding activity from IL-6 stimulated hepatocytes, capable of interacting with the acute phase response element (Raz et al., 1994). Further studies revealed that STAT3 acts as a latent transcriptional factor that mediates cellular response to diverse cytokines and growth factors in normal cells (Bowman et al., 2000). Aberrant activation of STAT3 is often associated with cell growth dysregulation and malignant cellular transformation (Bowman et al., 2000) and is found in a multitude of human cancers, including prostate (Mora et al., 2002), breast (Garcia et al., 2001), pancreatic (Scholz et al., 2003), and lymphomas (Zamo et al., 2002). In light of these findings, STAT3 is considered an oncogene. There is mounting evidence that the STAT3 signaling pathway is biologically important in the pathogenesis of cancer, however the mechanism

by which STAT3 activity contributes to oncogenesis have only partially been elucidated. Therefore, further work is required to further our understanding of the aberrant activation of the STAT3 signaling pathway in cancer. This can be done by identifying specific targets of STAT3 that may contribute to the pathogenesis of cancer, identifying novel stimulators that may contribute to STAT3 activation in cancer, and exploring the interaction of STAT3 with other oncogenes.

1.2. STAT family of transcription factors

There are seven members of the STAT family that have been identified: STAT1, 2, 3, 4, 5A, 5B, and 6, all of which are localized to three chromosomal clusters (Table 1) (Copeland et al., 1995; Ihle JN, 2001). STATs 1 and 4 are located on human chromosome 2 (murine chromosome 1); STAT2 and 6 are located on human chromosome 12 (murine chromosome 10); STAT3, STAT5A, and STAT5B are located on human chromosome 17 (murine chromosome 11) (Brierley and Fish, 2005; Ihle JN, 2001). STATs 1, 3, 4, 5A, and 5B are approximately 750 to 800 amino acids in length, whereas STATs 2 and 6 are approximately 850 amino acids long (Leonard and O'Shea, 1998). Amino acid sequences between the *STAT* genes have been evaluated and *STAT1/STAT4* show 50% similarity, *STAT2/STAT6* show 19% similarity, *STAT3/STAT5* show 24% similarity (Copeland et al., 1995). The similarities between the STAT family supports the concept that an initial *STAT* gene underwent tandem gene duplication, followed by subsequent duplication events (Copeland et al., 1995; Ihle JN, 2001; Leonard and O'Shea, 1998).

Table 1.1. STAT protein properties (adapted from Brierley *et al.*, Ihle *et al.*, Leonard and O'Shea, Lim and Cao)

Family member	Chromosomal location		Amino acid length	kDa	pTyr	pSer
	Murine	Human				
STAT1	1	2q12-33	750	91	Tyr701	Ser727
STAT2	10	12q13-14.1	851	113	Tyr690	-
STAT3	11	17q11.2-22	770	92	Tyr705	Ser727
STAT4	1	2q12-33	748	89	Tyr693	Ser721
STAT5A	11	17q11.2-22	794	94	Tyr694	Ser726/780
STAT5B	11	17q11.2-22	787	92	Tyr699	Ser731
STAT6	10	12q13-14.1	847	110	Tyr641	-

1.2.1. Domains of STAT proteins

Crystallographic studies of STAT1, STAT3, and STAT4 were first reported in 1998 (Becker et al., 1998; Chen et al., 1998; Vinkemeier et al., 1998). Figure 1.1a illustrates the three-dimensional structure of truncated STAT1 molecule bound to DNA with the truncated STAT3 molecule virtually superimposed with that of STAT1 (Chen et al., 1998). The crystal structures of STAT exhibit highly conserved structural features that are critical for STAT functions (Figure 1.1b) (Darnell, 1997). Beginning at residue 130 is the coiled-coil domain that consist of four anti-parallel long α-helices in which protein-protein interactions occur (Bromberg J and Darnell, 2000). This domain has been shown to mediate interaction between STAT1 and p48, a protein that belongs to an IFN response factor family, to form IFN-stimulated gene factor 3 complex (ISGF3) (Horvath et al., 1996). In addition, the first α-helix of the coiled-coil domain and a portion of the DNA-binding domain of STAT3 have been reported to interaction with the C-terminal region of c-Jun to activate transcription of α_2-macroglobulin (Zhang et al., 1999). Interestingly, it was also reported that the coiled-coil domain is essential for STAT3 recruitment to receptor, tyrosine phosphorylation, and subsequent tyrosine phosphorylation-dependent activities, including dimer formation, nuclear translocation, and DNA binding (Zhang et al., 2000). Furthermore, a recent report by Ma *et al.* identified Arg214/215 in the second α-helix of the coiled-coil domain and Arg414/417 in the DNA binding domain of STAT3 as sequence elements essential for nuclear translocation of STAT3 (Ma J, 2003).

The DNA binding domain, at residues 320-490, binds to DNA as a dimer and is also involved in nuclear translocation through association with protein import receptor, importin

(Ma and Cao, 2006). Despite no obvious sequence homology between STAT and other proteins, there is striking functional and structural similarities between STAT and Nuclear factor-κB (NF-κB) family of transcription factor where DNA contact is achieved by using loops protruding from a eight-stranded β-barrel domain with a variant of the immunoglobulin variable fold (Becker et al., 1998). STAT protein generally binds to palindromic sequence elements ($TTN_{(4-6)}AA$) that are related to the INF-γ activation sites. This consensus DNA element is referred to as the gamma interferon gene response (GAS) element, which reflects its initial characterization as a INF-γ activation sequence (Decker et al., 1991). The five base pair spacing was able to mediate transcriptional activation of STAT1 and STAT3 by IFN-γ, IL-6 and Oncostatin M (OSM) (Gearing et al.). However, testing of synthetic oligonucleotides bearing different STAT-binding sites revealed distinct binding specificities. For example, STAT1, STAT3, STAT4 and STAT5 favors a half-site spacing of three base pairs ($TTCN_3GAA$), whereas STAT6 prefers half-sites spaced by four base pairs ($TTCN_4GAA$) (Copeland et al., 1995; Seidel et al., 1995). A recent paper reported that specific STAT3-binding sites follow the sequence TTMXXXDAA (D: A, G, T; M: A, C) (Ehret et al., 2001; Qian et al., 2006). The differences in sequence specificity could be due to variations of specific DNA-contacting residues (Becker et al., 1998).

A linker domain from residues 390 to 580 is formed by two helix-loop-helix modules (helices α5 to α8) to separate the DNA-binding domain from the Src homology 2 (SH2) domain (Becker et al., 1998) and is involved in transcriptional activation in STAT1 by acting as a critical point for STAT1-driven transcription (Yang et al., 1999). Yang *et al.* reported that mutations within the linker domain destabilizes DNA binding and decreases

activation of genes after IFN-γ induction (Alli et al., 2002). Contrary to previous reports, the linker domain shows no structural similarity to SH3 domains (Becker et al., 1998).

Immediately downstream of the linker domain is the SH2 domain, an ~100 amino acid motif that extends between residues 580 and 680 and interacts with phosphotyrosine-containing motifs during signal transduction (Gao et al., 2004). The characteristic structure of the SH2 domain is a central three-stranded β-pleated sheet flanked by two α-helices (αββββα) with the first β-strand (βB) representing the core motif critical for phosphotyrosine (pTyr) binding and is required for normal function of the SH2 domain (Waksman et al., 1992). The SH2 domain is critical for receptor association and phospho-dimer formation in which homodimers as well as heterodimers between STAT1/STAT3 and STAT5A/STAT5B have been reported (Lim and Cao, 2006). Tyrosine phosphorylation of STAT3 occurs at a single site close to the carboxyl terminus (Tyr705) and serine phosphorylation of STAT3 occurs at a single residue (Ser727) (Wen and Darnell, 1997; Wen Z, 1995).

At one end of the STAT protein is the amino-terminal (N-terminal) domain (residues 1-130) and at the other end is the carboxyl-terminal (C-terminal) domain (starts at residues ~700). The N-terminal domain is essential for transcription, as judged by decrease stimulation in N-terminal deletion experiments (Meraz et al., 1996). There have also been reports of interactions between the N-terminal domain of STAT and histone acetyltransferase such as camp-responsive element binding protein (CBP/p300), which contributes to transcriptional activation (Zhang et al., 1996). CBP/p300 are important

transcriptional coactivators that interact with a wide range of DNA binding transcription factors to regulate cellular processes (Lejeune et al., 2002). In addition, most promoters have closely spaced tandem STAT binding sites and when two such sites are occupied, STAT dimer-dimer interaction occurs via the N-terminal domain. Alternatively, the C-terminal end of STAT is located downstream of the SH2 domain and functions as a transcriptional activation domain (TAD) (Darnell et al., 1994; King et al.). In general, the TAD of STATs can function independently by recruiting transcription co-activator CBP/p300 for gene activation (Paulson et al., 1999; Sun et al., 2006; Zhang et al., 1996). Specifically, STAT1, STAT3 and STAT4 TADs contains a conserved sequence motif in the C-terminal domain, Leu-Pro-Met-Ser-Pro (LPMSP), in which leucine phosphorylation as well as serine phosphorylation is required for maximal transcriptional activation (Wen et al., 1995). A recent report by Sun *et al.* suggests that although serine phosphorylation is necessary for maximal activity of STATs, it is not sufficient. Other residues in the LPMSP motif (Pro728 for STAT3) is required for maximal STAT-mediated transcriptional activation in response to cytokine signaling (Sun et al., 2006). In addition, transcriptional activation of STAT3β, a short form of STAT3 that lack a C-terminal region containing the LPMSP motif was significantly reduced (Caldenhoven et al., 1996). Figure 1.2 summarized the overall functions of the STAT domains.

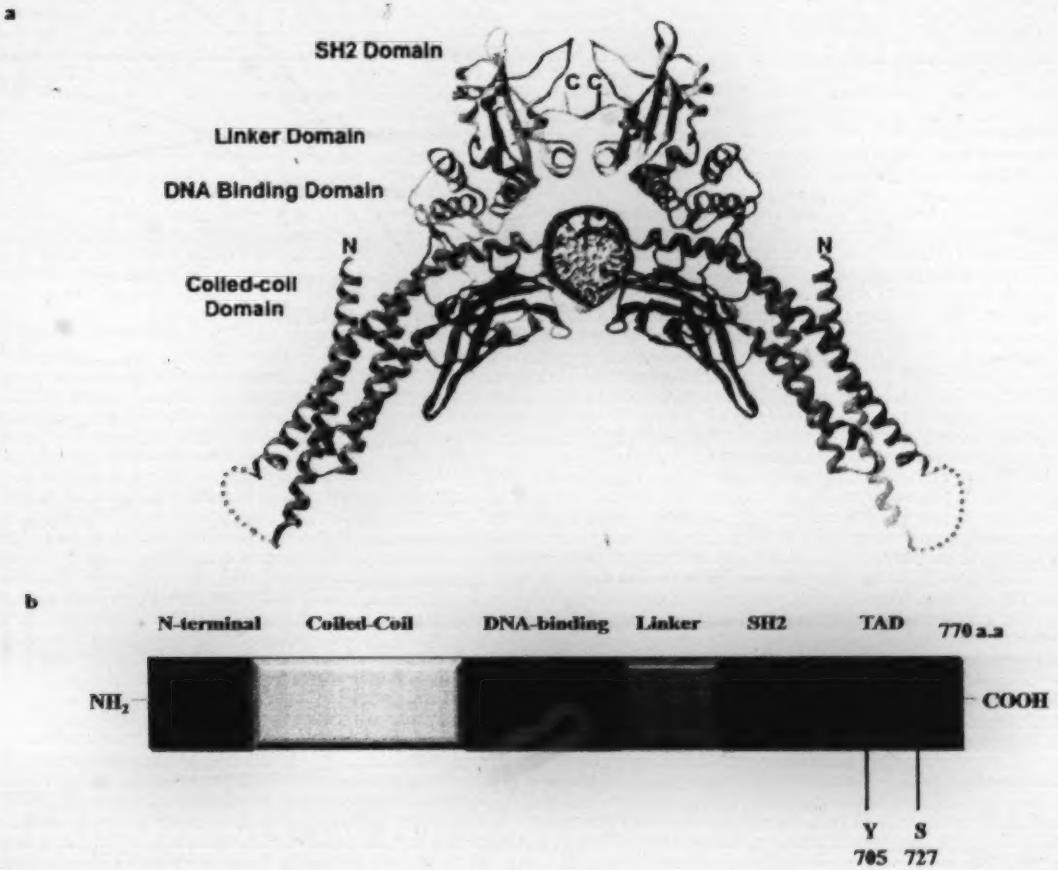

Figure 1.1. Structure of STAT3 protein. (a). Functional domain of STAT3 protein: N-terminal domain, Coiled-coil domain, DNA-binding domain, Linker domain, SH2 domain which is involved in receptor recruitment and STAT3 dimerization, and TAD domain. (adapted from Brierley *et al.* and Takeda *et al.*). (b). Crystal structure of a truncated STAT1 molecule bound to DNA. STAT3 truncated structure is virtually superimposed with the STAT1 structure. (adapted from Bromberg and Darnell)

Functional Domains	N		DNA	L	SH2	TAD
Receptor binding	-	+	-	-	+	-
Dimerization	+	-	-	-	+	+
Tetramerization	+	-	-	-	-	-
Nuclear import	+	+	+	-	-	-
Nuclear export	-	+	+	+	+	+
DNA binding	-	-	+	+	-	-
Transcriptional activity	-	-	-	+	-	+
Protein-protein interaction	+	+	+	+	+	+

Figure 1.2. STAT domain functions. The N-terminal domain is involved in dimerization, tetramerization, nuclear export, and protein-protein interaction. The coiled-coil domain is involved in receptor binding, nuclear import/export, and protein-protein interaction. The DNA-binding domain is involved in nuclear import/export, DNA binding, and protein-protein interaction. The linker domain is involved in nuclear export, DNA binding, transcriptional activity, and protein-protein interaction. The SH2 domain is involved in receptor binding, dimerization, nuclear export, and protein-protein interaction. Lastly, the transcriptional activation domain at the C-terminal end is involved in dimerization, nuclear export, transcriptional activation, and protein-protein interaction. (adapted from Lim *et al.*).

1.2.2. Isoforms of STAT proteins

The full length form of STAT proteins are referred to as the α isoform, whereas shorter β, γ, and δ isoforms were formed from either alternative splicing or post-translational proteolytic processing (Lim and Cao, 2006). Alternative spliced β isoforms with truncated C-terminal transactivation domains have been described for STAT1, STAT3, STAT4, STAT5A and STAT5B. Specifically, STAT3β lacks the 55 C-terminal amino acids of STAT3α but has a unique 7 amino acid sequence (Ren and Schaefer, 2002). Because of the carboxyl truncations, the β isoforms of STAT retain the critical phospho-Tyr residue but lack the phospho-Ser residue. Therefore, studies have reported that the β isoforms act as a dominant regulator of transcription (Caldenhoven et al., 1996). Furthermore, the α and β isoforms of STAT are distinctly different in their transcriptional activation and biological functions (Lim and Cao, 2006). In contrast to previous reports that STAT3β is a dominant negative factor, Maritano *et al.* reported that STAT3β can prevent embryonic lethality of STAT3-null mutations and can induce expression of STAT3 target genes (Maritano et al., 2004).

1.3. Regulation of STAT proteins

STAT activity is primarily regulated by post-translational modifications and a number of different post-translational modification mechanisms have been described to affect the regulation of STATs.

1.3.1. Phosphorylation

Tyrosine phosphorylation is considered to be obligatory for STAT dimerization and translocation into the nucleus (Calo et al., 2003). Interestingly, tyrosine phosphorylation is even critical for activation of the constitutive STATC mutants of STAT1 and STAT3 at A656C/N658C and A661C/N663C, respectively where mutations at cysteine residues resulted in spontaneous dimer-dimer interaction (Liddle et al., 2006). The critical tyrosine residues for STAT proteins are as follow: Tyr 701 for STAT1, Tyr 690 for STAT2, Tyr 705 for STAT3, Tyr 693 for STAT4, Tyr 694 for STAT5, and Tyr 641 for STAT6 (Table 1.1), and the resulting dimers are stabilized by divalent bonds (Chen et al., 1998; Liddle et al., 2006). All the STAT proteins are able to form homodimers except for STAT2 in which a heterodimer is formed with STAT1 and p48 (Horvath et al., 1996). Of all the tyrosine kinases, the one most studies is the Janus Kinase (JAK) family and this family of four mammalian kinases will be further discussed in chapter 1.5.3.

Aside from STAT2 and STAT6, in which tyrosine phosphoryalation is all that is required for activation, additional phosphorylation of the other STAT members at serine sites have been identified and extensively studied (Table 1.1) (Decker and Kovarik, 2000). Due to the presence of a Mitogen-activated protein kinase (MAPK) consensus sequence, Pro-Met-Ser-Pro (PMSP), in the C-terminal transactivation domain (residues 720 to 730) (Zhang et al., 1995), serine phosphorylation at Ser727 was described for STAT1 and STAT3 (Wen et al., 1995), and Ser721 was described for STAT4 (Visconti et al., 2000). In addition, STAT5A and STAT5B contain conserved Pro-Ser-Pro (PSP) motif with serine phosphorylation occurring at Ser725 and Ser779 in STAT5A and Ser730 in

STAT5B (Pircher et al., 1999; Yamashita et al., 1998). In light of these findings, it was suggested that members of the MAPK family participate in serine phosphorylation. For instance, phosphorylation can be achieved by a number of serine kinases including extracellular-signal-regulated kinase-1/2 (ERK1/2), c-Jun NH_2-terminal kinases (JNK), and p38 (Catlett-Falcone R, 1999; David et al., 1995b) where inhibition of the ERK pathway by PD98059 interfered with serine phosphorylation of STAT3 (Chung et al., 1997b). In addition, the contribution of these MAP kinases to serine phosphorylation is dependent on several factors including, the stimulus, cellular context, and STAT member. Phosphorylation of STAT3 at Ser727 by growth factors is attributed to ERKs (Chung et al., 1997b), whereas phosphorylation by IL-6 signaling is attributed to p38, not ERKs or JNK (Zauberman et al., 1999).

The role of serine phosphorylation on STAT members has been heavily studied with variable outcome. One major finding is that Ser727 phosphorylation of STAT1 and STAT3 is required for maximal transcriptional activation where mutation of Ser727 to Ala727 significantly reduced cytokine-stimulated transcription factor activity (Wen et al., 1995). It was also reported that the presence or absence of Ser727 phosphorylation did not affect tyrosine phosphorylation or DNA binding of STAT1 and STAT3 (Wen and Darnell, 1997). Although these findings have been disputed by other reports that showed that serine phosphorylation negatively regulates tyrosine phosphorylation and DNA binding (Chung et al., 1997b; Olayioye et al., 1999), a recent study by Shen *et al.* supports the concept that serine phosphorylation is essential for maximal transcription by demonstrating that mutation of the STAT3 Ser727 site in mice resulted in a 50%

transcriptional activation (Xie et al., 2006). Nevertheless, there are currently no evidence to support that serine phosphorylation can influence nuclear translocation of STATs and truncation of the C-terminal PMSP or PSP motif had no effect on the nuclear localization of STATs (Decker and Kovarik, 2000).

1.3.2. Arginine methylation, lysine acetylation, and ISGylation

In addition to tyrosine and serine phosphorylation of STATs, other post-translational modification includes methylation, ISGylation, ubiquitination, and SUMOylation. Methylation of STAT1 on arginine 31 in the N-terminal domain by protein arginine methytransferase 1 (PRMT1) is required to increase DNA binding activity and gene transcription. This is achieved by decreasing interaction with a member of the protein inhibitor of activated STAT (PIAS), PIAS1 (Mowen et al., 2001). Arginine methylation have also been reported for STAT3 (Rho et al., 2001) and STAT6 (Chen et al., 2004). In contrast, a recent report by Komyod *et al.* suggested that arginine methylation is absent in STAT1 and STAT3 due to the lack of methylation by PRMT1 *in vitro* (Komyod et al., 2005).

Reversible acetylation has been reported for STAT1, STAT3, and STAT6. Acetylation of STAT1 and STAT3 results in the activation of NF-κB, resulting in a pro-apoptotic effect for STAT1 and an anti-apoptotic effect for STAT3 (Kramer et al., 2006; Nadiminty et al., 2006). In addition, STAT3 acetylation at a single lysine residue, Lys685, is critical for dimer-dimer formation, cytokine-stimulated DNA binding, and transcriptional activation (Liu et al., 2005a).

Emerging evidence suggests that protein modification by ISG15 (ISGylation) positively regulates IFN signaling to enhance and prolong JAK-STAT3 signaling. Thus far, only STAT1 have been reported to be a target for ISGylation (Malakhova et al., 2003). Following IFN stimulation, the ISGylating enzyme Ubc8 conjugates ISG15 to signaling components such as STAT1 and though it appears to act as a positive regulator of STAT1 activity, ISGylation is not essential for JAK-STAT signaling (Leslie et al., 2006). Therefore, the biological significance of ISGylation on STAT regulation needs to be further studied.

1.4. Nuclear trafficking of STAT3 protein

Eukaryotic cells have evolved with the genomic information partitioned from the cytoplasm by a membrane bound nucleus. The trafficking of molecules in and out of the nucleus occur through specialized passageways known as nuclear pore complexes (NPCs) that span the membrane (Rout et al., 2000; Suntharalingam and Wente, 2003). Small molecules can diffuse freely through the NPCs, but larger molecules need to interact with transport carrier proteins to pass through the NPCs. The recognitions signals on protein cargos are specific amino acid sequences that can either function as nuclear-localization signals (NLSs) or nuclear–export signals (NESs). The transport carriers are members of the karypherin-β that play a primary role in nuclear importing and exporting and therefore are referred to as importins and exportins, respectively. Karyopherin-β proteins can bind protein cargo directly or require interaction with adaptor molecules such as the importin-α family. Figure 1.3 illustrates the nuclear import and export mechanism of STAT3.

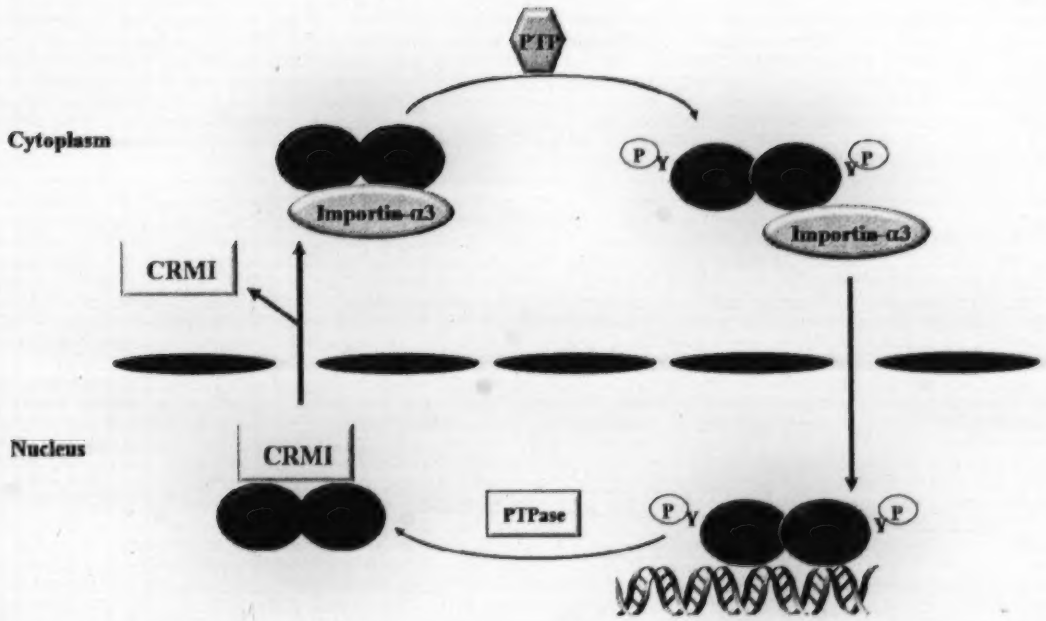

Figure 1.3. Schematic illustration of STAT3 protein import/export cycle. Importin-α3 binds to the NLS in STAT3 and mediates nuclear accumulation of both unphosphorylated and tyrosine phosphoryated STAT3 (by protein tyrosine kinases). The nuclear export of STAT3 is mediated by unknown exportin and is also facilitated by interaction of CRM1 with putative NESs (via protein tyrosine phosphatases) (adapted from Reich and Liu).

1.4.1. Nuclear import

Analysis of endogenous STAT3 using immunofluorescence clearly shows the constitutive presence of STAT3 in the nucleus (Liu et al., 2005b). Unlike the other STAT family members, STAT3 can accumulate in the nucleus independent of the phosphorylation state, as demonstrated by mutating the tyrosine and arginine residue in the SH2 domain (Tyr705 to Phe and Arg609 to Ala, respectively) (Liu et al., 2005b; Reich and Liu, 2006). STAT3 have intrinsic constitutive domains that allows for continuous shuttling of STAT3 between the nucleus and cytoplasm, with nuclear import being more dominant (Liu et al., 2005b; Reich and Liu, 2006). Nuclear translocation of STAT3 is mainly mediated by importin-α3, a member of the importin-α family that is ubiquitously expressed (Kohlhase et al., 1996) and interacts with the coiled-coil domain of STAT3 at amino acids 152-163. In addition, this particular amino acid sequence is required by the STAT3 constitutive NLS (Liu et al., 2005b). Importin-α3 binds to the constitutive NLS in STAT3 and mediates nuclear accumulation of both unphosphorylated and tyrosine phosphoryated STAT3 (Reich and Liu, 2006).

Since there is a prominent amount of unphosphorylated STAT3 that can accumulate in the nucleus, a recent report proposed that unphosphorylated STAT3 can influence gene expression independently or through interaction with other DNA-binding factors, (Yang et al., 2005). Further work have revealed that unphosphorylated STAT3 can regulate expression of a variety of genes by interacting with NF-κB (Yang et al., 2007). In addition to its interaction with NF-κB, interaction between STAT3 and other transcription factors such as Jun have cooperatively influenced gene transcription (Zhang

et al., 1999). Overall, unphoshorylated STAT3 can be continuously shuttled between the nucleus and cytoplasm, thereby allowing for rapid response to activating signals (Reich and Liu, 2006)..

1.4.2. Nuclear export

Many properties of nuclear imports are shared with the nuclear exports of STAT3. For active export of molecules the presence of NES is required. A common NES is a hydrophobic sequence that is rich in leucine amino acids and recognized by the exportin transporter chromosome region maintenance 1 (CRM1). CRMI can bind to protein containing NES and to Ran-GTP in the nucleus. Once transported to the cytoplasm, Ran-GTP is hydrolyzed and CRM1 is dissociated from the protein (Reich and Liu, 2006). For STAT3 nuclear export, three NES elements at amino acids 306-318, 404-414, and 524-535 were identified and functional studies using green fluorescent protein (GFP)-tagged reporter proteins at leucine-rich sequences confirmed that the three elements did in fact direct the CRM1-dependent nuclear export (Bhattacharya and Schindler, 2003). It was suggested that the structural changes that are associated with STAT3 activation may be involved in regulating NES functions due to the fact that one of the hydrophobic residues is inaccessible or only partially exposed (amino acids 404-414 and 524-535, respectively) (Becker et al., 1998; Bhattacharya and Schindler, 2003). In contrast, the STAT3 NES at amino acids 306-318 is more accessible in the activated conformation and appears to be more important in postreceptor-stimulated export (Bhattacharya and Schindler, 2003).

1.5. Activation mechanisms of STAT3 proteins

STAT proteins acts as a latent transcriptional factor that mediates cellular response to diverse cytokines and growth factors in normal cells (Bowman et al., 2000). There are multiple tyrosine kinase signaling pathways involved in STAT3 activation. Generally, stimulation of cell surface receptors with their cognate ligands results in activation of receptor associated tyrosine kinases, such as the JAK, which will autophosphorylate at tyrosine docking sites. STAT3 monomers will bind to JAK via the SH2 and is phosphorylated at the Tyr705 residue. This phosphorylated form of STAT3 will dimerize and translocate to the nucleus to modulate transcription of various targets genes that are often key regulators of cell survival, cell cycle progression, and apoptosis (Bromberg J and Darnell, 2000; Levy and Lee, 2002). Alternatively, STAT3 can also be activated by non-receptor tyrosine kinases (Figure 1.4). One interesting feature of STAT3 activation is that STAT3 DNA binding activity can be detected in the nucleus within minutes of cytokine stimulation. Thus, the acronym 'STAT' is very appropriate since it reflects the speediness by which STATs become activated to exert its biological functions (Leonard and O'Shea, 1998).

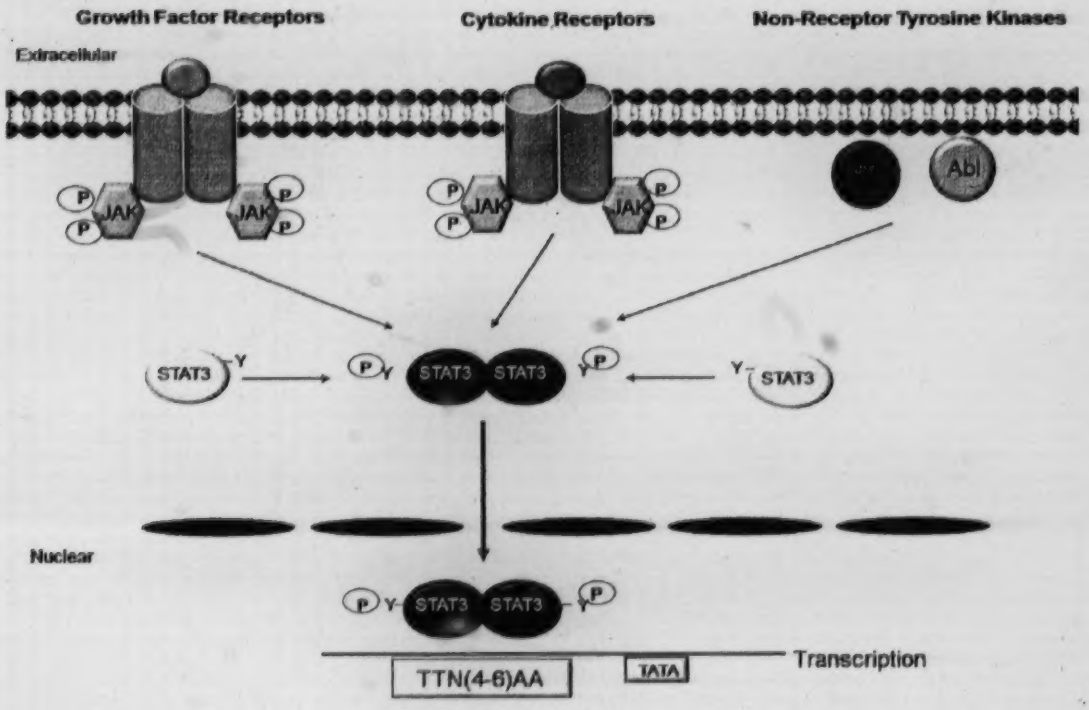

Figure 1.4. Activation mechanisms of STAT3. Multiple tyrosine kinase signaling pathways are involved in STAT3 activation. First, STAT3 can be activated by cytokines (i.e. IL-6) that can directly bind to receptors lacking intrinsic tyrosine kinase activity. This results in phosphorylation and activation of appropriate JAK family kinases. JAK will then phosphorylate STAT3 protein resulting in dimerization, nuclear translocation and DNA binding. Second, STAT3 can be activated by growth factors (i.e. EGF) that bind to receptor tyrosine kinases. This will also result in the activation of STAT3 through JAK. Lastly, STAT3 can also be activated by non-receptor tyrosine kinases (i.e. Src). These non-receptor tyrosine kinases can directly phosphorylate STAT3 in the absence of ligand-induced receptor signaling. (adapted from Bowman *et al.*).

1.5.1. Cytokines

STAT3 is considered a critical mediator of biological responses promoted by a variety of cytokines (Table 1.2). Receptors that binds to cytokines constitutes a large number of factors that are essential in regulating multiple aspects of cell growth and can be divided into two groups (Calo et al., 2003). Class I includes receptors for IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, IL-15, erythropoietin (Epo), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), granulocyte and granulocyte/macrophage colony stimulating factor (G-CSF and GM-CSF) (Copeland et al., 1995). This group of receptors shares a domain with conserved motifs containing four cysteine and one tryptophan residues and a membrane proxial region encoding tryptophan-serine-X-tryptophan-serine (WSxWS) where 'X' is a non-conserved residue (Calo et al., 2003). Although class II receptors share overall structural features with class I receptors, they are more divergent. Class II receptors, including ones for IFN-α, INF-γ, and IL-10, encode several conserved pralines and tyrosines as well as an additional cysteine pair (Calo et al., 2003; Ghosh et al., 1993; Novick et al., 1994). Cytokine receptors have no intrinsic enzymatic activity and therefore determine STAT phosphorylation in tyrosine by means of proteins belonging to the JAK family (Takeda K, 2000).

Table 1.2. Activation of STAT proteins by various ligands (Adapted from Schindler and Darnell)

	STATs					
Ligands	1	2	3	4	5	6
IFN family						
IFN-α	+	+	+	-	+	
IFN-γ	+	-	-	-		
IL-10	+/-	-	+	-		
gp130 family						
IL-6	+/-	-	+	-	-	-
IL-11	+/-	-	+	-	-	-
OSM	+/-	-	+	-	+/-	-
LIF	+/-	-	+	-	+/-	-
CNTF	+/-	-	+	-	-	-
G-CSF	+	-	+	-	+	
IL-12			+	+	-	-
γ-C family						
IL-2	+/-		+		+	
IL-4	+		+		+	+
IL-7		+	+	+	+	+
IL-9	+		+		+	
IL-13						+
IL-15		+	+		+	+
1L-21	+		+		+	
gp140 family						
IL-3					+	
IL-5					+	
GM-CSF	+		+		+	
Growth hormone family						
EPO	+	-	+	-	+	
GH	+	-	+	-	+	
PRL	+	-	+	-	+	
Receptor tyrosine kinases						
EGF	+	-	+	-	+	
PDGF	+	-	+	-	+	
HGF	+	+	+		+	
CSF-1	+		+			

The activation of STAT3 by the IL-6 family of receptors have been well documented (Lutticken et al., 1994; Smith et al., 1988; Zhong et al., 1994). Members of this family, including IL-6, IL-11, OSM, LIF, and CNTF, all share a common gp130 receptor component (Copeland et al., 1995; Lutticken et al., 1994; Smith et al., 1988; Zhong et al., 1994). Binding of the IL-6 family cytokines to their receptors leads to either gp130 homodimerization or heterodimerization of gp130 with other related receptors, including IL-6 receptor α, LIF receptor (LIFR)-β, OSM receptor-α, and CT-1 receptor-α. This subsequently results in the activation of the gp130-associated JAK family members (Lutticken et al., 1994; Sato et al., 2003; Smith et al., 1988) and phosphorylation of tyrosine residues located in YxxQ boxes (Tyr767, 814, 905, and 915) creates docking sites for STAT3 (Figure 1.5) (Heinrich et al., 1998). These docking sites for STAT3 significantly contributes to IL-6 mediated STAT activation by allowing JAKs to phosphorylate STAT3 at Tyr705 in the SH2 domain, leading to STAT3 dimerization, and translocation to the nucleus (Copeland et al., 1995).

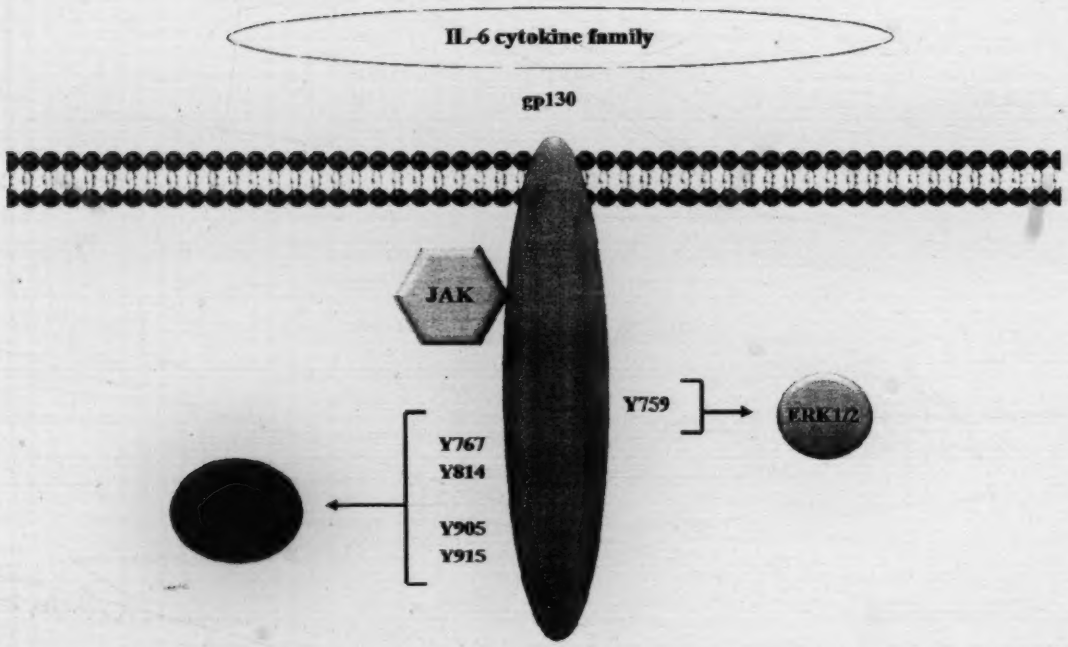

Figure 1.5. Phosphorylation of JAK by gp130. Stimulation of gp130 will result in the phosphorylation of Y759, Y767, Y814, Y905, and Y915 by JAK family kinases. Of the five tyrosine sites, Y767, Y814, Y905, and Y915 are required for STAT3 activation. In contrast, Y759 is required for ERK1/2 kinase activation. (adapted from Hirano *et al.*).

1.5.2. Growth factors

STATs are also activated by several receptors for growth factor such as EGF (Leaman et al., 1996), PDGF (Vignais et al., 1996), hepatocyte growth factor (HGF) (Boccaccio C, 1998), and colony stimulating factor 1 (CSF-1) (Novak et al., 1995) (Table 1.2). These receptors possess intrinsic tyrosine kinase activities and can therefore activate STATs indirectly, by means of JAK kinase proteins, or directly, as demonstrated by activation of STATs by EGF or PDGF in the absence of the JAK family members (David et al., 1996; Leaman et al., 1996; Vignais et al., 1996). For example, EGF receptor (EGFR) is a membrane-bound receptor tyrosine kinase that initiates activation of a number of signal transduction pathways that are critical for cell proliferation and survival, including STAT3 (Jin L, 1997). EGFR mediated activation of the gp130/JAK/STAT3 pathway via IL-6 up-regulation have recently been reported (Berishaj et al., 2007).

1.5.3. Janus Kinase family

Four mammalian members of JAK have been identified using two approaches. One member, TYK2, was identified by screening of a T cell cDNA libraries at low-stringency hybridization using a c-fms catalytic domain (Krolewski et al., 1990). The other three members, JAK1, JAK2, and JAK3, were cloned using primers corresponding to conserved motifs within the catalytic domains of tyrosine kinases (Harpur et al., 1992; Rane and Reddy, 1994; Wilks et al., 1991). While JAK1, JAK2 and TYK2 are ubiquitously expressed, JAK3 expression in tissues is more restricted (Leonard and O'Shea, 1998). The JAK family represents a distinct family of receptor-associated tyrosine kinases which have been implicated in the signal transduction of many members

of the cytokine family (Figure 1.6) (Copeland et al., 1995). The physical association between JAKs and cytokine receptors was demonstrated by interaction of JAK2 and EPO (Argetsinger et al., 1993). As previously mentioned, cytokine binding to its receptor activates the appropriate JAKs, leading to tyrosine phosphorylation of receptors which serves as docking sites for STAT3 via the SH2 domain (Imada K, 2000). In addition, different classes of receptors preferentially use one particular JAK or a combination of JAKs (Figure 1.5). For instance, growth hormone and EPO receptors employ JAK2, whereas IL-6 receptors use JAK1, JAK2, and TYK2. In contrast, JAK3 is only activated by cytokines whose receptors contain γ-chain: IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 (Ahr et al., 2005; Leonard and O'Shea, 1998; Murray, 2007). Moreover, a recent study reported the activation of JAK3 by IL-9 and γ-chain, which led to the subsequent activation of STAT3 in a type of lymphoma (Qiu et al., 2006).

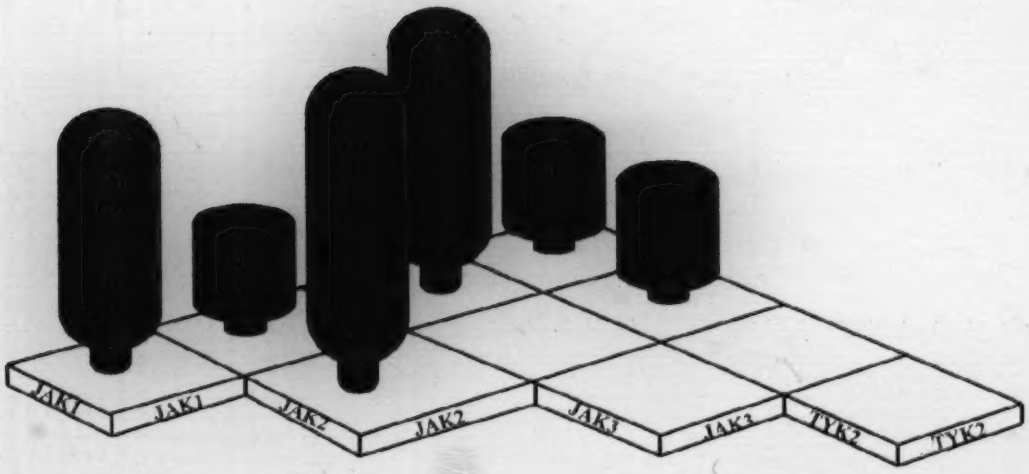

Figure 1.6. JAK combinations utilized by cytokine receptors. Most cytokine receptors use three different JAK combinations. Receptors required for hemopoietic cell development and proliferation utilizes JAK2, common γ-chain receptors utilizes JAK1 and JAK3, and other receptors utilize only JAK1. Receptors that use JAK2 and JAK3, JAK3 alone, TYK2 alone, or JAK3 and TYK2 are still unknown. (adapted from Murray *et al.*, Leonard *et al.*).

1.5.4. Non-receptor tyrosine kinases

Several oncoproteins of the non-receptor tyrosine kinases have been identified which activates STAT3. STAT3 activity has been associated with Src where cellular transformation of mammalian cells by Src reveals constitutive activation of STAT3. Furthermore, blockade of STAT3 using dominant negative construct was sufficient in blocking the transforming ability of Src (Turkson et al., 1998). Src is the first tyrosine kinase that was discovered and can interact with STAT3 independently or through the EGF and PDGF (Levy and Darnell, 2002). Another example is Abl, an oncoprotein that can directly interact with JAKs to activate STAT3. In contrast, the fusion protein breakpoint cluster region-Abl (BCR-Abl) can directly activate STAT3 without JAK tyrosine kinase activity. Other examples of viral oncoproteins including, v-Eyk, v-Ros, v-Fps, and Lck have been known to activate STAT3 in predominantly fibroblast cell lines (Bowman et al., 2000).

1.6. Negative regulation of STAT3

STAT3 activation is normally a transient process and inactivation or inhibition of STAT3 is important to control their biological functions. Several distinct negative regulators of STAT3 signaling pathway have been defined.

1.6.1. Cytoplasmic tyrosine phosphatases

As crucial as tyrosine kinases are in initiating STAT3 signaling, tyrosine phosphatase is essential for the abrogation of signal transduction (Brierley and Fish, 2005). Several protein tyrosine phosphatases (PTPs) that can dephosphorylate STATs in the cytoplasm

have been described, including SH2-containing phosphatase-1 (SHP1), SHP2, and protein-tyrosine-phosphatase-1B (PTP1B) (Aoki and Matsuda, 2000; David et al., 1995a; You et al., 1999). SHP1 is a non-transmembrane PTP that is abundantly expressed in hematopoietic cells (Yi et al., 1992) and represses signaling through several receptors, including EPO, IL-3, IL-4, IL-13, and CSF-1 (Shultz et al., 1997). SHP1 serves as an important negative regulator of the STAT3 signaling pathway, as a loss of SHP1 in anaplastic lymphoma kinase positive anaplastic large cell lymphoma (ALK^+ALCL) increases tyrosine phosphorylation and activation of the JAK3/STAT3 signaling pathway and decreases proteosome degradation of JAK3 (Han et al., 2006). On the other hand, SHP2 is ubiquitously expressed and is reported to reduce tyrosine phosphorylation of mainly STAT1 and STAT5 (Brierley and Fish, 2005). PTP1B is expressed ubiquitously and absence of PTP1B in mice leads to impaired JAK2 dephosporylation which results in leptin hypersensitivity and enhanced STAT3 tyrosine phosphorylation (Zabolotny et al., 2002).

1.6.2. Nuclear regulators

STAT dephosphorylation by nuclear PTPs occurs in the nucleus and the half-life for nuclear dephosphorylation of STAT proteins is approximately 10-15 minutes (Zabolotny et al., 2002). The nuclear tyrosine phosphatase TC45 is a nuclear isoform of the T-cell protein tyrosine phosphatase (Tc-PTP) that is implicated in the dephosphorylation of both STAT1 and STAT3, since dephosphorylation of STAT1 and STAT3 is defective in Tc-PTP null mouse embryonic fibroblasts (ten Hoeve et al., 2002). Furthermore, TC45 has also been implicated in dephosphorylating JAK1 and JAK3 (Simoncic et al., 2002).

PIAS are a family of nuclear proteins that are constitutively expressed and bind phosphorylated STAT dimers to inhibit their activity. Four members of PIAS have been identified, including PIAS1, PIAS3, PIASx, and PIASy (Brierley and Fish, 2005). The PIAS family of proteins have several conserved domains: the SAF-A, Acinus, PIAS (SAP) box required for repression of STAT1 by PIASy (Liu et al., 2001), the Miz-Zn finger/RING domain that is essential for small-ubiquitin like modifier (SUMO) ligase activity (Smith et al., 2004), and the PINIT motif for nuclear retention of PIAS3 (Duval et al., 2003). Studies in cultured mammalian cells confirmed that PIAS1 and PIAS3 only interacted with tyrosine phosphorylated STAT1 and STAT3, respectively (Chung et al., 1997a; Liu et al., 1998). PIAS3 is constitutively expressed in a large variety of tissues and is reported to block the DNA-binding activity of STAT3 to inhibit activation of target genes (Chung et al., 1997a). A recent report revealed a short amino acid sequence from the N-terminal region of PIAS (residues 82 to 132) that is able to bind and inhibit STAT3 activity (Levy and Inghirami, 2006). In contrast, PIASx and PIASy do not inhibit DNA-binding activity of STATs but rather act as transcriptional co-repressors. For example, PIASy blocks STAT1 mediated transcription but not DNA-binding (Choi et al., 2001).

1.6.3. Suppressor of cytokine signaling

Another distinct family of negative regulators of STAT is the suppressor of cytokine signaling (SOCS) proteins. SOCS family protein consist of eight members, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, SOCS7, and cytokine inducible SH2 domain-containing (CIS), that act through JAKs or receptors to block STAT activation (Brierley and Fish, 2005). All of the SOCS proteins are characterized by a central SH2 domain, a

N-terminal domain of variable length and sequence, and a C-terminal region containing a conserved SOCS box (Greenhalgh and Hilton, 2001). The SOCS proteins are generally induced in response to cytokine stimulation and can bind to the activated signaling components to inhibit signaling directly or by targeting the active proteins for ubiquitin/proteosome-mediated degradation (Krebs and Hilton, 2001). Within the SOCS family, SOCS1 and SOCS3 have been shown to be the most potent regulators of cytokine signaling (Brierley and Fish, 2005). Knockout studies revealed that SOCS1 is important in regulating IFN-γ signaling and STAT1 activation (Alexander et al., 1999). In addition, SOCS1 and SOCS3 appear to play a role in inhibiting STAT3 activity due to the absence of IL-6 dependent stimulation of STAT3 in cells expressing these SOCS proteins (Nicholson et al., 1999). In particular, the negative effects of SOCS3 on STAT3 signaling have been extensively studied. Under physiologic conditions, IL-6 induces the expression of SOCS3 via the JAK/STAT3 pathway, which will allow SOCS3 protein to bind to gp130 at Tyr759, resulting in the inhibition of IL-6 signaling and STAT3 activation (Croker et al., 2003; Heinrich et al., 1998; Kubo et al., 2003). Therefore, the silencing of SOCS3 is considered a potential mechanism to explain the constitutive activation of STAT3 in a variety of human cancers (Flowers et al., 2005; Niwa et al., 2005; Weber et al., 2006). One possible reason for the silencing of SOCS3 in cancer is the aberrant methylation of the CpG islands, as reported in human hepatocellular carcinoma cell lines (Niwa et al., 2005).

1.7. Biological functions of STAT3

Activation of STAT3 leads to transcriptional induction of genes that evoke a number of cellular functions including cell proliferation, differentiation, survival, and development, as well as specialized functions that involve immune responses (Bowman et al., 2000). In this section of chapter 1, the multi-functional role of STAT3 will be discussed.

1.7.1. Requirement of STAT3 for embryonic development

In contrast to the other STAT family members, STAT3 knockout mice result in embryonic lethality (Table 1.3) (Takeda et al., 1997). Similar to the wild-type embryos, $STAT3^{-/-}$ embryos developed to the egg cylinder-stage embryos until 6.0 days post coitum (dpc). However, the embryos and the inner cell mass (ICM) of the $STAT3^{-/-}$ are much smaller then the wild-type embryos with rapid degeneration occurring between 6.5 and 7.5 dpc. Expression of STAT3 mRNA was observed exclusively at around 6.0 dpc in the visceral endoderm, which plays an important supportive role during early embryogenesis by mediating metabolic exchange with maternal blood (Takeda et al., 1997). Therefore, the coincidence of the onset of degeneration of $STAT3^{-/-}$ embryos with STAT3 mRNA expression indicates that embryonic lethality may be due to a defect in functions of visceral endoderm, such as nutritional insufficiency (Takeda et al., 1997). Gene deletion of various receptors that are known to activate STAT3, including gp130, LIFR, and CNTF receptor (CNTFR), were also analyzed with embryonic lethality observed. However, the period of embryonic lethality in $STAT3^{-/-}$ mice is shorter then what was observed for the receptors with $LIFR^{-/-}$ mice and $CNTFR^{-/-}$ mice dying shortly after birth and $gp130^{-/-}$ mice dying around 15.5 to 18.5 dpc (Bowen et al., 1995; DeChiara

et al., 1995; Yoshida et al., 1996). Therefore, these components do not explain the reason behind the early embryonic lethality observed after knockdown of STAT3. Overall, the process by which STAT3 mediates embryonic development remains to be determined.

Table 1.3. STAT knockout studies (Adapted from Levy and Darnell)

STAT family member	Phenotype of knockout mice
STAT1	Impaired responses to INF, increased susceptibility to tumors, viable, normal development
STAT2	Impaired responses to INF, viable, normal development
STAT3	Early embryonic lethality, impaired cell survival in adult tissues, impaired response to pathogens
STAT4	Impaired TH1 response, loss of IL-12 responsiveness
STAT5A	Impaired mammary gland development
STAT5B	Impaired growth hormone responsiveness
STAT6	Impaired TH1 differentiation, loss of IL-4 responsiveness

1.7.2. Conditional knockout of STAT3

Because knockdown of the *STAT3* gene resulted in lethality at such an early stage, it was critical to development conditional knockout mice to study STAT3-dependent processes in later stages of embryogenesis and beyond. The Cre-loxP recombinase system was applied where deletion of the DNA segment flanked by the *lox*P sites, following the introduction of Cre recombinase, results in functional inactivation of the *STAT3* gene (Levy and Lee, 2002). Table 1.4 summarizes the tissue specific role of STAT3 using conditional knockout mice.

Using the lymphocyte specific protein tyrosine kinase (*Lck*) promoter to drive Cre, the function of STAT3 in T-cells were examined (Akaishi et al., 1998; Takeda et al., 1998). STAT3-deficient T-cells showed severely impaired IL-6 dependent T-cell proliferation (Takeda et al., 1998) and displayed a reduced IL-2 dependent T-cell proliferation due to impaired IL-2 receptor α chain expression (Akaishi et al., 1998). STAT3 functions have also been investigated in macrophages and neutrophils using the macrophage lysozyme (*Mlys*) promoter to direct Cre-mediated gene ablation (Chapman et al., 1999). STAT3-deficient macrophages and neutrophils showed increased production of inflammatory cytokines, including tumor necrosis factor (TNF)-α, IL-6, and IFN-γ, and enhance major histocompatibility complex (MHC) class II and B7-1 co-stimulatory molecule. In addition, IL-10 mediated suppression of macrophages and neutrophils were completely abolished (Chapman et al., 1999). The role of STAT3 in the skin have also been evaluated using the keratin 5 (*K5*) promoter and hair cycle and wound healing were severely compromised due to defective keratinocyte motility (Toi M, 1998).

Furthermore, STAT3 knockout studies have also been performed in mammary gland using the β-lactoglobulin (*Blg*) promoter (Chapman et al., 1999). In normal mammary glands, high levels of phosphorylated STAT3 levels are detected during puberty, a time of rapid ductal growth and branching, and early pregnancy (Hennighausen et al., 1997). However, levels of STAT3 are diminished during lactation and are only induced shortly after the cessation of suckling, during a phase called involution. Involution is characterized by extensive apoptosis of the epithelial cells alongside an increase of insulin-like growth factor binding protein 5 (IGFBP5), a protein that is responsible for inducing apoptosis (Takeda K, 2000). Therefore, STAT3 gene ablation in mammary gland delayed involution after weaning and decreased apoptosis of the epithelial cells. IGFBP5 expression was almost abolished but other markers of apoptosis, including Bax and Bcl-xL, were unaffected (Chapman et al., 1999)

Table 1.4. STAT3 conditional knockout study (Adapted from Levy and Darnell, Levy and Lee)

Target cells	Phenotype
T lymphocytes	Impaired IL-6 dependent survival and IL-2 receptor expression
Monocytes/neutrophils	Increased inflammatory cytokines and TH1 differentiation
Keratinocytes	Impaired 2nd hair cycle, wound repair, and keratinocyte motility
Mammary epithelium	Delayed involution, defective apoptosis
Thymic epithelium	Thymic hypoplasia dependent on age, hypersensitivity to stress
Liver	Impaired acute phase response

1.7.3. Embryonic stem cell

Embryonic stem (ES) cells are pluripotent cell lines that are derived from the inner cell mass of preimplantation embryo and can differentiate into various organs derived from the three embryonic germ layers: endoderm, mesoderm, ectoderm (Evans and Kaufman, 1981; Martin, 1981; Rossant and Papaioannou, 1984). ES cells are considered totipotent where the cells have the ability to give rise to all of the somatic cells as well as germ cells (Evans and Kaufman, 1981; Hahn et al., 1999). The presence of LIF is compulsory to maintain ES cells in a self-renewal state and prevent differentiation (Smith et al., 1988; Smith and Hooper, 1987; Williams et al., 1988); this will activate the gp130 signaling pathways, resulting in STAT3 phosphorylation. STAT3 have been implicated as a major player in maintaining the pluripotency and self-renewal of murine ES cells since a decrease in STAT3 activation will result in loss of pluripotency and enhanced cell differentiation (Raz et al., 1999; Takeda et al., 1997). This finding contradicts the involvement of STAT3 as an inducer of differentiation in somatic cell types and provides further evidence that interpretation of STAT3 activation is cell-type specific (Niwa et al., 1998). Several transcription factors, including Oct4, Sox2, Nanog, and SALL4, also play a fundamental role in the self-renewal and pluripotency of mouse ES cells (Chambers and Smith, 2004; Sakaki-Yumoto et al., 2006). Furthermore, a number of these transcription factors are known to interact with STAT3 (Chambers, 2004). A recent study by Torres *et al.* demonstrated the collaboration between STAT3 and Nanog to promote self-renewal of ES cells (Torres and Watt, 2008).

1.8. Role of STAT3 in oncogenesis

In normal cells, STAT3 activation is a tightly controlled mechanism that allows the cell to respond appropriately to cellular stimulus. In contrast, aberrant activation of STAT3 has been increasingly associated with malignant cell growth. Yu *et al.* provided the first evidence of STAT3 playing a critical role in cancer by demonstrating the constitutive activation of STAT3 in cells transformed by oncoprotein Src (Yu et al., 1995). The finding that ablation of STAT3 signaling prevents the transformation of fibroblasts by Src further confirmed the definitive role of STAT3 in oncogenesis (Bromberg et al., 1998; Turkson et al., 1998). Subsequently, several other tyrosine kinase pathways were reported to activate STAT3 and contribute to oncogenesis (Garcia et al., 1997). Importantly, constitutive activation of STAT3 have also been linked to transformation by oncoproteins, including human T-lymphotropic virus-1 (HTLV-1) (Russell et al., 1995), polyomavirus middle T antigen (Garcia et al., 1997), and Epstein-Barr virus (EBV) (Weber-Nordt et al., 1996), that can activate JAKs or Src family tyrosine kinases. In contrast, examples of tyrosine kinase signaling pathways that do not play a role in STAT3-dependent cellular transformation, includes v-Ras and v-Raf (Garcia et al., 1997).

Essential evidence for a key role for STAT3 in oncogenesis was first described in multiple myeloma (Catlett-Falcone R, 1999). Since then, aberrant activation of STAT3 have been observed in a wide variety of human cell lines and primary tumors, including leukemias, lymphomas, breast cancer, head and neck cancer, pancreatic cancer, ovarian cancer, and prostate cancer (Table 1.5) (Bowman et al., 2000; Bromberg, 2002; Garcia et al., 2001; Huang et al., 2000; Lin et al., 2000). The mechanism of STAT3 activation has

been elucidated in a variety of human cancers. For instance, activation of STAT3 in head and neck squamous cell carcinoma was mediated by EGFR and tumor growth factor (TGF)-α and targeting of EGFR and TGF-α decreased STAT3 activity and tumor growth (Grandis et al., 1997; Grandis et al., 1998; Grandis and Tweardy, 1993). In addition, a decrease in SOCS1 activity was responsible for abnormal expression of STAT3 in hepatocellular carcinoma (Lejeune et al., 2002), and the IL-6 signaling pathway is implicated in the constitutive STAT3 activity in some acute myeloid leukemia (AML) cases (Yoshikawa et al., 1999). Although it is highly likely that there are a variety of mechanisms involved in generating constitutively active STAT3, the basis for this activation is still not fully understood.

Bromberg *et al.* provided further evidence for the role of active STAT3 in oncogenesis by using a constitutively active mutant STAT3 molecule (Bromberg et al., 1999). The constitutively active STAT3 molecule, termed STAT3C, was genetically engineered by replacing two amino acids in the SH2 domain for cysteine residues. The cysteine residues are capable of dimerization in the absence of tyrosine phosphorylation, and will migrate to the nucleus, bind to STAT3 promoter regions, and induce gene expression. FLAG, an epitope tag was also appended for ease in following the molecule in cells. FLAG is a hydrophilic purification tag consisting of 8 amino acids that was specifically designed for antibody-mediated identification and purification of recombinant proteins (Einhauer and Jungbauer, 2001). Notably, the introduction of STAT3C into fibroblast sufficiently transformed the cells, as demonstrated by the formation of tumors in

immunodeficient mice. Furthermore, STAT3C was capable of activating a number of known STAT3 target genes (Bromberg et al., 1999).

Table 1.5. STAT activation in human tumors and cell lines (Adapted from Garcia and Jove, Hodge *et al.*)

Tumor type	Activated STAT
Breast cancer	
Tumors	STAT1, STAT3
Cell lines	STAT3
Multiple myeloma	
Tumors and cell lines	STAT1, STAT3
Head and neck cancer	
Tumors and cell lines	STAT1, STAT3
Leukemias (Tumors and cell lines)	
Acute lymphocytic leukemia	STAT1, STAT5
Acute myelogenous leukemia	STAT1, STAT3, STAT5
Chronic myelogenous leukemia	STAT5
Large granular lymphocyte leukemias	STAT3
Chronic lymphocytic leukemia	STAT1, STAT3
Lymphomas (Tumors and cell lines)	
Anaplastic large cell lymphoma	STAT3, STAT5
EBV-related Burkitt's lymphoma	STAT3
Cutaneous T cell lymphoma	STAT3
Prostate	
Tumors and cell lines	STAT3
Renal cell carcinoma	
Tumors and cell lines	STAT3
Pancreatic adenocarcinoma	
Tumors and cell lines	STAT3
Ovarian carcinoma	
Tumors and cell lines	STAT3
Melanoma	
Tumors and cell lines	STAT3

1.9. Target genes regulated by STAT3

Aberrant activation of STAT3 may contribute to oncogenesis by activating a variety of key gene products that are compulsatory for initiation and maintenance of malignant transformation (Bowman et al., 2000). Previous studies to identify specific STAT3 target genes revealed that STAT3 regulates expression of genes that are involved in cell cycle progression, apoptosis, angiogenesis, cell invasion, metastasis, and immune function (Table 1.6) (Dauer et al., 2005; Galkin et al., 2007).

STAT3 regulates the expression of genes involved in cell cycle progression, including cyclin D1 and c-Myc (Kiuchi et al., 1999; Leslie et al., 2006; Sinibaldi et al., 2000). Cyclin D1 can control cell progression from G1 to S phase in STAT3C expressing cell by interacting with cyclin-dependent kinase (CDK)-4 and CDK6 (Bromberg et al., 1999). In addition, STAT3 can transcriptionally regulate cyclin D1 by binding to the *cyclin D1* promoter region (Leslie et al., 2006). Furthermore, STAT3-mediated expression of cyclin D1 is required for anchorage-independent growth where STAT3C expression increased anchorage-independent growth in cyclin D1 wild type, but not in cyclin D1 -/- cells (Leslie et al., 2006). Interestingly, expression of cyclin D1 is a result of v-Src induced STAT3 activation in NIH3T3 fibroblast cells (Sinibaldi et al., 2000). STAT3 have also been reported to regulate c-Myc, a proto-oncogene found to be overexpressed in a number of human malignancies (Dang et al., 1999). c-Myc is a transcription factor that plays a critical role in regulating cell proliferation and cell survival (Bowman et al., 2000). Elevated mRNA levels of c-Myc have been reported in fibroblast cells stably expressing STAT3C (Bromberg et al., 1999) and c-Myc expression is induced in

response to STAT3 signaling in v-Src transformed NIH3T3 fibroblasts (Bowman et al., 2001). Furthermore, STAT3 binds to a consensus sequence on the *c-Myc* promoter to drive transcriptional activation of c-Myc in response to IL-6 with ablation of the STAT3-binding site on the *c-Myc* promoter being sufficient in abolishing IL-6-induced transcription of c-Myc (Kiuchi et al., 1999). Interestingly, c-Myc is a negative regulator of $p21^{WAF1/CIP1}$ transcription and is stimulated by Src to bind to the $p21^{WAF1/CIP1}$ promoter and prevent its activation. $p21^{WAF1/CIP1}$ is a CDK2 inhibitor that contains three STAT binding sites on its promoter region, two for STAT1 and one for STAT3 (Chin et al., 1996). In addition, $p21^{WAF1/CIP1}$ can interact with STAT3 at the *c-Myc* promoter to inhibit c-Myc expression (Vigneron et al., 2005).

STAT3 is also implicated as a critical regulator of apoptosis and a number of anti-apoptotic genes in the B-cell lymphoma-2 (Bcl-2) family, including Bcl-2, Bcl-xL, and myeloid cell leukemia-1 (Mcl-1) are responsive to STAT3 (Bromberg et al., 1999; Catlett-Falcone R, 1999). For instance, Bcl-xL expression is directly controlled by STAT3 signaling in a human myeloma cell line and blocking of STAT3 activity suppressed Bcl-xL expression and subjected the cells to Fas-mediated apoptosis (Catlett-Falcone R, 1999). Similarly, shutdown of STAT3 activity can efficiently decrease Bcl-xL expression and increase tumor cell apoptosis in head and neck squamous cell carcinoma. Furthermore, a recent study demonstrated that NF-κB and STAT3 are co-activators that can modulate the expression of Bcl-xL as well as pro-apoptotic protein Bax. Inhibition of NF-κB and STAT3, alongside re-expression of tumor suppressor p53, was sufficient in decreasing Bcl-xL and increasing Bax expression in head and neck

squamous cell carcinoma (Lee et al., 2008). Mcl-1, another anti-apoptotic protein, is also regulated by STAT3 signaling in such cancer cells as leukemic large granular lymphocytes and shutdown of STAT3 activity resulted in decreased Mcl-1 expression and restored sensitivity of the cells to Fas (Epling-Burnette et al., 2001). Consistent with these findings, STAT3 have been shown to directly bind to consensus binding sequence on the *Mcl-1* promoter and disruption of the STAT3-binding sites decreased *Mcl-1* promoter activity (Isomoto et al., 2005).

STAT3 have also been linked with target genes that are involved in angiogenesis, cell invasion, and immune function (Table 1.6). Angiogenesis is a complex, multistage process that involves endothelial cell proliferation, degradation of basement membrane and extracellular matrix, endothelial cell migration, and formation of tubular structure (Folkman, 2003). STAT3 plays a role in modulating angiogenesis through the regulation of a number of angiogenic factors, including vascular endothelial growth factor (VEGF) (Niu et al., 2002). STAT3 can also regulate VEGF expression indirectly by inducing the expression of hypoxia-inducible factor-1α (HIF-1α) and basic fibroblast growth factor (bFGF), both of which are key regulators of VEGF (Xie et al., 2006; Xu et al., 2005). Although STAT3 has been shown to regulate the genes involved in promoting cell invasion, including matrix metalloproteinases (MMP)-2, MMP-1, MMP-9 (Dechow et al., 2004; Itoh et al., 2006; Xie et al., 2004), and inhibitors of cell invasion, such as tissue inhibitor of metalloproteinases (TIMP)-1 (Bugno et al., 1995), the exact role for STAT3 in modulating invasion is incompletely understood and will be assessed further in chapter 2. Finally, STAT3 have also been reported to play a pivotal role in regulating tumor

immunity. STAT3 activation in tumor cells inhibits expression of inflammatory cytokines and chemokines, such as IFN-β and TNF- α (Huang, 2007). Moreover, STAT3 appears to play a role in tumor evasion where blockade of STAT3 in the immune cells of tumor-bearing hosts resulted in an increase in anti-tumor immune response, including dendritic cells, T-cells and natural killer cells (Kortylewski et al., 2005). Although it is clear that STAT3 regulates multiple genes that could contribute to oncogenesis, it is highly likely that there are other unknown STAT3 target genes that may also contribute to oncogenesis. Therefore, the work in this thesis further investigated novel stimulators of the STAT3 signaling pathway in oncogenesis.

Table 1.6. STAT3-regulated target genes (Adapted from Yu *et al.*)

Gene function	Target gene
Proliferation and survival	cyclin D1
	Myc
	p21
	Bcl-xl
	Mcl-1
	Survivin
	p53
Angiogenesis/cell invasion	VEGF
	HGF
	HIF-1
	MMP-2
	MMP-1
	MMP-9
	TIMP1
Immunosuppression	TGF
	INF
	IL-10
	IL-6
	IL-12
	TNF

1.10. STAT3 activation in human cancer

Aberrant STAT3 activation has been implicated in a variety of human tumors, including blood malignancies and solid tumors (Bowman et al., 2000; Bromberg, 2002; Garcia et al., 2001; Huang et al., 2000; Lin et al., 2000). The scope of the work for this thesis was performed using two different human cancer models, both of which are known to have high STAT3 activation (Watson and Miller, 1995; Zamo et al., 2002).

1.10.1. Breast cancer

Breast cancer is the most common cancer among Canadian women today despite advances in diagnosis, prevention and treatment (Garcia et al., 1997). Recent studies have reported the constitutive activation of STAT3 in breast cancer cell lines and approximately 50% of primary breast tumors, but not in benign tumors (Diaz et al., 2006; Dolled-Filhart et al., 2003; Gritsko et al., 2006; Leslie et al., 2006; Turkson J, 2000; Vultur A, 2004). Activation of STAT3 leads to transcriptional induction of target genes that evoke a number of cellular functions such as cell proliferation, transformation, and migration (Silver et al., 2004; Wei et al., 2003). This may be implicated in the pathogenesis of breast cancer. For example, EGF promotes the growth of breast cancer cells and the activation of STAT3 in breast cancer cell lines (Berclaz G, 2001), and EGFR and Src activity correlates with STAT3 in breast cancer cells. STAT3-dependent overexpression of Bcl-2 protein also contributes to chemotherapy resistance in breast cancer cell lines (Real et al., 2002). Mutations of the breast cancer susceptibility gene 1 (BRCA1), directly activates STAT3, resulting in an increased cellular proliferation and inhibition of apoptosis (Gao et al., 2001). Mucin 1 (MUC1), a transmembrane molecule

that play a role in cell adhesion, is associated with metastatic potential and invasion of breast cancer cells and is shown to carry the STAT3 responsive element in the gene promoter and can be up-regulated *in vitro* (Gaemers et al., 2001). Moreover, STAT3 modulates survivin, a known effector of cell survival and angiogenesis (Leslie et al., 2006). In contrast, dominant-negative inhibition of STAT3 has been sufficient in blocking proliferation and survival of breast cancer cells *in vitro* (Burke et al., 2001; Garcia et al., 2001; Li and Shaw, 2002). Although constitutive STAT3 activation in breast cancer have been extensively studied, the exact biological role and effect of STAT3 is incompletely understood.

1.10.2. Anaplastic lymphoma kinase-positive Anaplastic large cell lymphoma

ALK^+ALCL is defined as a subtype of T/null-cell non-Hodgkin lymphoma characterized by the consistent expression of CD30. ALCL comprises 2 to 8% of non-Hodgkin lymphomas in adults and 10 to 15% in children, making it one of the most common forms of pediatric cancer (Amin and Lai, 2007). Doxorubicin-containing combination chemotherapy induces complete remission in up to 95% of patients with relapse occurring in greater than 40% of cases, making ALK^+ALCL clinically aggressive (Amin and Lai, 2007). The aberrant expression of ALK in approximately 80% of ALK^+ALCL tumors is a result of the reciprocal chromosomal translocation, t(2;5)(p23;q35), which leads to the fusion of the nucleophosmin (*NPM*) gene at *5q35* with the Anaplastic lymphoma kinase *(ALK)* gene at *2p23* (Morris et al., 1994; Shiota et al., 1994a). ALK is a transmembrane receptor tyrosine kinase belonging to the insulin receptor superfamily and its expression in humans are normally limited to rare cells of neural origin (Fujimoto

et al., 1996). In contrast, NPM is ubiquitously expressed and function as a shuttling protein between the nucleus and cytoplasm (Amin and Lai, 2007). The fusion protein, nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) has been shown to be oncogenic, and it directly contributes to the pathogenesis of ALK^+ALCL through exerting its constitutively active tyrosine kinase embedded in the ALK portion of the fusion protein (Slupianek et al., 2001). While NPM-ALK has been shown to activate multiple signaling pathways (Slupianek et al., 2001), there are accumulating evidence supporting the concept that NPM-ALK mediates its oncogenic effects via STAT3 activation (Chiarle et al., 2005; Khoury JD, 2003; Zamo et al., 2002). Aberrant activation of STAT3 is highly consistent in ALK^+ALCL, and transfection of ALK^+ALCL cell lines with a STAT3 dominant negative construct resulted in cell cycle arrest and apoptosis (Khoury JD, 2003). STAT3 is key mediator in ALK^+ALCL, making it a perfect study model to further our understanding of STAT3 in cancer.

1.11. Cancer therapeutics for STAT3

For years, the standard treatment of cancer has been intravenous cytotoxic chemotherapy. However, these drugs target cancer cells as well as normal tissues resulting in toxicity (Gerber, 2008). In the past 10 years, identification of key targets in cell signaling has led to a dramatic shift in cancer therapies with the emergence of target-directed therapies (Galkin et al., 2007). For instance, STAT3 integrates signals from a variety of stimuli and pathways and regulates genes that are involved in key cellular processes. Therefore, several possible anti-cancer strategies have been pursued to target the STAT3 signaling

pathway, including receptor antagonists, tyrosine or serine kinase inhibitors, and direct inhibition of STAT3 (Al Zaid Siddiquee and Turkson, 2008).

Inhibition of JAK family kinases using AG490 and prevention of STAT3 binding to EGFR using peptides are examples of therapeutic strategies currently pursued, and have been shown to have growth inhibitory effects on epithelial and hematologic tumors (Buerger et al., 2003; Burke et al., 2001; De Vos et al., 2000). Furthermore, Src family kinase inhibitors have been shown to block STAT3 activation and induce apoptosis in breast cancer cell (Garcia et al., 2001). Therapeutic approaches have also been made to inhibit STAT3 target genes such as cyclin D1 where treatment with proteosome inhibitor bortezomib can inhibit STAT3 expression in cyclin D1 overexpressing breast cancer cells (Asao et al., 2001). Examples of direct inhibitors of STAT3 includes, G-quartet oligonucleotides which have been utilized to form complex tertiary structures and inhibit STAT3 dimerization and cancer cell growth (Jing et al., 2004). In addition, double-stranded and single-stranded oligonucleotides can be used to compete with STAT3 for binding to their cognate DNA sequences (Barton et al., 2004). Small-molecule inhibitors, including JSI-124, WP1066 and STA-21, have also been one of the strategies used to inhibit STAT3 signaling (Huang, 2007). For instance, STA-21 is hypothesized to bind to the SH2 domain of STAT3 to block subsequent dimerization of STAT3 (Bhasin et al., 2008). Overall, targeting STAT3 protein for therapeutic intervention still remains to be fully explored and investigation of novel stimulators and targets of the STAT3 signaling pathway may allow for the development of new therapeutic strategies.

1.12. Thesis overview and objectives

Signal transduction, defined as cellular signals that modulate cellular behavior or function (Galkin et al., 2007) are being increasingly studied to explore and possibly elucidate the mechanism of carcinogenesis. Since its discovery, an extensive amount of experimental research and clinical observations have confirmed that STAT3 is an oncogene. However, the mechanistic role of STAT3 in tumorigenesis is biological complex, where extensive crosstalk between multiple networks are involved in the oncogenic process. Since STAT3 is a multifaceted regulator of transcription and mediator of oncogenesis, further studies are required to fully understand the biological impact of STAT3 activation in cancer.

The scope of this thesis is to increase our understanding of the role of STAT3 in the pathogenesis of cancer and to demonstrate that STAT3 is biological and clinically significant in the pathophysiology of a variety of human cancer types. The overall objectives of this thesis is to increase our understanding of the effects of STAT3 in cancer by identifying specific targets of STAT3 that may contribute to the pathogenesis of cancer, identifying ESPs and corresponding receptors that may contribute to STAT3 activation in cancer, and exploring the possible interactions of STAT3 with other oncogenes.

In chapter 2, the link between TIMP1, a protein that is known to regulate cell migration and metastasis, and STAT3 in breast cancer cell lines was investigated and the biological and clinical significance of STAT3 activation and TIMP1 expression in breast cancer was

assessed. In chapter 3 and 4, two novel upstream stimulators of STAT3 in ALK^+ALCL was investigated for its contribution to tumorigenicity. In chapter 5, a novel biological relationship between STAT3 and SALL4, a novel transcriptional factor found to be constitutive active in hematological malignancies was identified. In conclusion, using two different human cancer models, I have furthered our understanding of how STAT3 contributes to oncogenesis by identifying novel upstream stimulators and target genes of STAT3. This allowed me to further delineate possible mechanisms involved in STAT3 activation and the effects of STAT3 activation in cancer, providing a framework for novel strategies of targeting STAT3 signaling in cancer.

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Chapter 2 ♦

STAT3 Upregulates TIMP1 Expression and Decreases Invasiveness of Breast Cancer

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2.1. Introduction

STATs are a family of latent transcriptional factors that become activated in response to cytokines and growth factors (Burke et al., 2001; Hsiao et al., 2003). Ligand-dependent activation of STATs, which is a transient process lasting for minutes to hours, plays important roles in embryogenesis, cell proliferation and differentiation (Aoki et al., 2003; Bromberg et al., 1999). However, abnormal activation of STAT3 has been shown to contribute to deregulation of cell growth and malignant cellular transformation in many types of human cancers, and there is mounting evidence to support that STAT3 is an oncogene (Bromberg et al., 1999; Burke et al., 2001; Catlett-Falcone R, 1999; Levy and Lee, 2002; Ni et al., 2000). Many STAT3 downstream target genes have been identified in various cell types, including cell cycle facilitators cyclin D1 and c-Myc, as well as anti-apoptotic proteins such as survivin, Bcl-xL, Mcl-1, and Bcl-2 (Bromberg, 2002; Huang et al., 2000). STAT3 has been reported to be aberrantly activated in 30 – 60% of primary breast cancer, as shown by the presence of STAT3 tyrosine phosphorylation and DNA-binding (Clevenger, 2004; Dechow et al., 2004; Watson, 2001). In one study, STAT3 activation was found to be associated with a better clinical outcome (Dolled-Filhart M, 2003). With this background, it is likely that aberrant activation of STAT3 is important in the pathogenesis of breast cancer. Nevertheless, the exact biological role of STAT3 in breast cancer is not fully understood.

TIMPs constitute at least four distinct members, TIMP1, 2, 3, and 4, which have been shown to inhibit tumor invasion and metastasis, by inhibiting MMPs (Fassina et al.,

2000). TIMP1, a 28 kDa sialoglycoprotein (Ritter et al., 1999), is regulated by a number of cytokines and growth factors such as IL-6 (Bugno et al., 1995), TNF-α, and TGF-β (Kossakowska et al., 1999). TIMP1 is a potent inhibitor of MMP-2 and MMP-9, and has been shown to prolong survival in animals xenografted with tumors (Zacchigna et al., 2004). In line with these findings, it has been shown by immunohistochemical analysis that TIMP1 overexpression in breast cancer cells may be an indicator of favorable prognosis in breast cancer patients (Nakopoulou et al., 2003). On the other hand, TIMP1 has been shown to possess anti-apoptotic properties in some cancer cell types, including breast cancer (Catlett-Falcone R, 1999; Liu et al., 2005c; Stetler-Stevenson et al., 1997). A number of previous studies also have shown that high serum levels of TIMP1 are associated with poor prognosis in patients with gastric carcinoma (Yoshikawa et al., 1999) and colorectal carcinoma (Pellegrini et al., 2000). Thus, the biological roles and clinical significance of TIMP1 appear to be cancer cell-type specific.

Recent studies have suggested that STAT3 protein may play a role in regulating TIMP1 expression, as the promoter of the *TIMP1* gene contains STAT3 binding sites (Bugno et al., 1995). Our lab previously showed that inhibition of STAT3 signaling using a dominant negative construct effectively downregulates TIMP1 expression in ALK^+ALCL cell lines, and expression of TIMP1 in this type of lymphoma in fact correlates with the STAT3 activation status (Lai et al., 2004). With this background, I hypothesize that STAT3 activation may modulate tumor invasiveness of breast cancer by regulating TIMP1 expression. In this study, I investigated the link between TIMP1 expression and STAT3 activation in breast cancer cells, and assessed the biological and clinical

significance of STAT3 activation and TIMP1 expression in breast cancer. An *in vitro* model was created that involved breast cancer cell lines transfected with STAT3C in which expression can be regulated by the addition of tetracycline (i.e. tet-off system). Using this model, the link between STAT3 activation status and TIMP1 expression was established in breast cancer cells. The effects of STAT3 activation and TIMP1 expression on invasiveness was also investigated using matrigel invasion assays. To validate these *in vitro* data, STAT3 activation and TIMP1 expression in 142 primary breast cancer tissues was examined and correlated with the presence or absence of lymphatic and vascular invasion.

2.2. Materials and Methods

2.2.1. Construction of tet-off STAT3C

STAT3C construct is a generous gift from Dr. Bromberg, and its properties have been well characterized and described (Bromberg et al., 1999). Briefly, the constitutively dimerizable form of STAT3 was created by substituting cysteine residues for specific amino acids within the C-terminal loop of the SH2 domain, allowing for sulfhydryl bonds to form between the STAT3 monomers and rendering the molecule capable of spontaneous dimerization without phosphorylation at Tyr705. Thus, the STAT3C dimer is capable of translocating to the nucleus and driving gene transcription. Due to its ability to transform cells and promote tumor formation, STAT3C has been widely used to alleviate the importance of STAT3 activation in human cancers. To create tet-off STAT3C, we first synthesized Tetracycline response element (TRE)-STAT3C, which was generated by digesting the 2.8 Kb fragment of STAT3C from the STAT3C plasmid

using *Bam*H1. The STAT3C fragment was then ligated with the pTREzhyg vector (Clontech, Mountain View, CA, USA), which was then transformed into *E.coli* and selected on ampicillin resistant plates. The plasmids were isolated using the mini-prep kit (Qiagen Science, Mississauga, ON, Canada). Orientation and integrity of the generated TRE-STAT3C plasmid was analyzed by DNA sequencing using the 3130XL Genetics Analyzer (Applied Biosystems, Foster City, CA, USA). The TRE-STAT3C plasmid was amplified using the Maxiprep kit (Qiagen).

2.2.2. Cell lines and Culture

Human breast cancer cell lines MCF-7 (ATCC HTB-22) and MDA-MB-436 (ATCC HTB-130) and grown at 37°C in 5% CO2. MCF-7 was maintained in Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) and MDA-MB-436 was maintained in RPMI-1640 (Sigma-Aldrich). Both types of culture media were enriched with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and antibiotics (10,000 units/mL penicillin G, 10,000 µg/mL streptomycin, Gibco). All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). To create permanent cell clones, MCF-7 and MDA-MB-436 were transfected with tetracycline-controlled transactivator (tTA) plasmid (Clontech). Cell clones were selected and maintained by the addition of G418 (800 and 100 µg/mL, respectively) (Gibco) to the culture media. Cell clones permanently transfected with tTA will be subsequently transfected with TRE-STAT3C to create the tet-off STAT3C system.

2.2.3. Transfection

Transient transfection of breast cancer cell lines, MCF-7 and MDA-MB-436, with the various vectors was performed using Lipofectamine Transfection Reagent (Invitrogen Life technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, breast cancer cell lines were grown in 6-well culture plates (Bectin Dickinson Labware, Meylan Cedex, France,) without antibiotics. When cells reached approximately 80% confluency, culture media was replaced with serum-free Opti-MEM I (Gibco), and the cells were transfected with DNA:Lipofectamine complex. To obtain the tTA permanent cell clones, breast cancer cell lines were transfected with 1 μg of plasmid tTA, followed by antibiotic selection. Permanent breast cancer cell clones expressing tTA were also transiently transfected with 1 μg of plasmid TRE-STAT3C. To regulate the expression of STAT3C in these cells, various concentrations of tetracycline (ng/mL) (Invitrogen) were added to the cell culture.

2.2.4. Western Blot Analysis and Antibodies

Western blot analysis was performed using standard techniques. Briefly, 24 hours after transfection, cells were harvested by gentle scraping and washed twice in Phosphate-Buffer Saline (PBS) (Sigma). Lysis buffer (20 mM tris-hydrochloride (HCL), pH 7.5, 5.0 mM EDTA, 40.0 μg/mL leupeptin, 1 μM pepstatin, 1 mM AEBSF) was added to the cell pellet for 30 minutes on ice followed by centrifugation at 14,000*g* for 10 minute at 4°C. The supernatant was removed and 50-100 μg of protein was run on SDS-PAGE. Protein quantification was carried out using the Bradford assay according to the manufacturer's protocol (Biorad). After the proteins were transferred to nitrocellulose membranes, the

membranes were blocked with 5% milk in Tris-Buffer Saline (TSB) (Sigma) and 0.05% Tween-20 (Calbiochem, Gibbstown, NJ, USA), and then incubated with primary antibodies overnight at 4°C followed by 1 hour incubation with horseradish peroxidase-conjugated secondary antibody (1:10000) (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). The membranes were washed in TBS with 0.05% Tween-20 for 30 minutes between steps. Proteins were detected using the enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, IL, USA). The number in parenthesis after each antibody listed indicates the dilution at which it was used. Antibodies used included: mouse monoclonal anti-human FLAG (1:3000, Sigma), rabbit polyclonal anti-human STAT3 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-human pSTAT3 (1:500, Santa Cruz), mouse monoclonal anti-human TIMP1 (1:500, Santa Cruz), mouse monoclonal anti-human cyclin D1 (1:1000, Santa Cruz), mouse monoclonal anti-human Bcl-2 (1:1000, Santa Cruz), mouse monoclonal anti-human Bcl-xL (1:500, Santa Cruz), and mouse monoclonal anti-human β-actin (1:3000, Sigma-Aldrich).

2.2.5. Immunofluorescence staining and confocal microscopy

Immunofluorescence was performed using standard techniques. Briefly, cells grown on coverslip at 80% confluency in a 6-well plate were fixed with 4% paraformaldehyde in PBS. Cells were rinsed with 1X PBS, permeabilized with PBS-0.5% triton X-100 for 5 minutes, and rinsed twice with 1X PBS. Cells were incubated with 25 µL of anti-STAT3 antibody for 30 minutes followed by rinsing once with 1X PBS-triton (0.1%) and twice with 1X PBS. After Incubation with 25 µL of Alexa 488 goat anti-mouse secondary

antibody for 30 minutes and repeated rinsing, mounting media containing DAPI (Sigma-aldrich) was added to the slides. Cells were visualized and imaged with a Zeiss LSM 510 confocal microscope (Oberkochen, Germany). Argon laser with a 488 nm wavelength was used to visualize STAT3 at 40X objective and images were analyzed using the Zeiss LSM 5 image browser.

2.2.6. Matrigel Invasion Assay

Prior to use, matrigel chambers (BD Biosciences, Bedford, MA, USA) were kept at room temperature for 1 hour. 500 µL of PBS was added to the inserts and incubated at 37°C in 5% CO_2 for 2 hours for equilibration. During incubation, MCF-7 cells transfected with tTA-TRE-STAT3C and treated with various concentrations of tetracycline was trypsinized and washed three times with sterile PBS. Cells were resuspended to 25,000 cells in 500 µL of DMEM without phenol red since phenol red is known to interfere with the assay. After 2 hours of equilibration, the 500 µL of PBS was aspirated from the inserts and replaced with the 500 µL cell suspension. 750 µL of DMEM containing 5% FBS was added to the bottom of the wells to serve as chemo-attractants. The plate was incubated for 24 hours at 37°C in 5% CO_2. Following incubation, the cells on the upper surface of the insert were removed by gentle scraping with sterile cotton swab. Invasive cells that migrated to the lower side of the insert were fixed with methanol and stained with trypan blue solution (Sigma). Five random 40X fields were counted per sample and each sample was performed in triplicates. The matrigel invasion assay was also performed using transfected cells treated with TIMP1 neutralizing antibody (Acris Antibodies, Hiddenhausen, Germany).

2.2.7. ELISA Assay for TIMP1

MCF-7 and MDA-MB-436 cells transfected with tTA-TRE-STAT3C were treated with increasing concentrations of tetracycline and incubated at 37°C in 5% CO_2. At 24, 48 and 72 hours incubation periods, aliquots of medium were extracted, spun at 14,000 rpm, and supernatant was collected and stored at -80°C. 100 µL of sample and was placed in microtiter wells (Oncogene Research Products, Cambridge, MA, USA), covered and incubated for 30 minutes at 4°C. Each well was washed four times with 1X wash buffer (Oncogene Research Products), and 100 µL of TIMP1 conjugate was added to each well, followed by incubation for 30 minutes at 4°C. A color reagent was then added to each well and incubated in the dark at room temperature for 30 minutes. The color reaction was stopped by 2.5 N Sulfuric Acid (Oncogene Research Products), and a colorimetric plate reader measured each absorbance at 450/595 nm. A standard curve for the assay was generated using the reconstituted standards provided by the kit and absorbances of the test samples were interpreted by reading directly from the standard curve concentrations.

2.2.8. Immunohistochemistry

Immunohistochemical staining was performed using standard techniques. Briefly, formalin-fixed, paraffin-embedded tissue sections of 4 µM thickness were deparafinized and hydrated. Heat-induced epitope retrieval was performed using Tris buffer (pH 9.9; Dako, Mississauga, Ontario, Canada) and a rapid microwave histoprocessor (RHS, Milestone, Bergamo, Italy). After incubation at 100°C for 10 minutes, slides were washed in running tap water for 5 minutes, followed by a wash

with PBS (pH 7.2) for 5 minutes. Tissue sections were then incubated with mouse monoclonal anti-human pSTAT3 antibody (1:60) (Santa Cruz) or mouse monoclonal anti-human TIMP1 antibody (1:200) (Santa Cruz) overnight in a humidified chamber at 4°C. After 3 washes with PBS, tissue sections were incubated with secondary antibody, anti-rabbit IgG and peroxidase (Dako) for 30 minutes at room temperature. The tissue sections were incubated with 3,3'-diaminobenzidine/H_2O_2 (Dako) for color development, using hematoxylin as a counterstain. To optimize the IHC staining, normal breast tissues were used as positive controls and benign tonsils were used as negative controls. Two pathologists, who were blinded to the results of the other markers, examined the immunohistochemical staining. For pSTAT3, the presence of any areas with >10% tumor cells showing definitive nuclear staining were scored positive. Endothelial cells that are often positive for pSTAT3 served as internal positive controls whereas reactive lymphoid cells in benign tonsils served as negative controls. As for TIMP1, the presence of any areas with definitive cytoplasmic staining in >10% tumor cells was considered positive. Reactive germinal centers served as positive controls and the mantle zone B-cells served as negative controls. Discrepancies of the immunostaining results between the two observers were reconciled on a multi-headed microscope.

2.2.9. Patient and Tumor Specimens

Archival, formalin fixed, paraffin-embedded tissues from the 142 cases of primary breast cancer reviewed at the Cross Cancer Institute evaluated between 1996 and 2001 was randomly chosen for this study. This study has been previously reviewed and

approved by the institutional ethics committee. The initial diagnoses of all cases were made at the community hospitals, which were subsequently reviewed by a pathologist at the Cross Cancer Institute according to a standardized institutional guideline. The histologic grades of these 142 cases were as follows: grade 1 (20 cases), grade II (42 cases) and grade III (80 cases). The clinical stages of these patients are summarized as follows: stage IIA (75), stage IIB (45), stage IIIA (2), stage IIIB (18), and stage IV (2). The median age of patients was 61 years (range, 35 to 90 years).

2.2.10. Statistical Analysis

Graphpad Instat 3 software was used for statistical analysis of the data. The association between STAT3, TIMP1 and lymphatic/vascular invasion was evaluated by Chi square test. The survival data were analyzed using the Logrank test and Kaplan-Meier survival curves were generated. A P value <0.05 was considered statistically significant. Each assay was performed at least in triplicate and the illustrative graphs are representative of the mean of the triplicate experiments. Error bars represent standard error of mean (SEM).

2.3. Results

2.3.1. Characterization of MCF-7 transfected with tTA-TRE-STAT3C

We first evaluated the *in vitro* model in which the expression of STAT3C can be regulated by addition of various concentrations of tetracycline to the cell culture. As described in Methods and Materials, MCF-7 cells were initially transfected with tTA. In

the tet-off system, tTA binds to the TRE sequence to activate transcription of specific genes. This transcriptional activation can be controlled by the addition of tetracycline, which will bind tTA and render it incapable of binding to the TRE sequence (Gossen and Bujard, 1992). Once the permanent cell clones expressing tTA were established, the cells were then transfected with TRE-STAT3C and treated with increasing dosages of tetracycline for 24 hours. Expression of the transgene (i.e. STAT3C) was primarily assessed by evaluating the expression of FLAG, the epitope tagged to the STAT3C construct. Western blot analysis revealed a gradual decrease in the expression of FLAG with addition of tetracycline in a dose dependent fashion in MCF-7 cells (Figure 2.1). The FLAG epitope, which is only associated with the exogenous but not the endogenous STAT3, became completely undetectable at 60 ng/mL. At 60 ng/mL of tetracycline, the total STAT3 band was decreased to a level similar to that of MCF-7 cells transfected with tTA alone.

The properties of STAT3C have been extensively characterized and published (Bromberg et al., 1999). To further confirm that STAT3C is biologically active, Western blots were performed to correlate the expression of a known STAT3 downstream target with various expression levels of STAT3C. Treatment of MCF-7 transfected cells with increasing concentrations of tetracycline resulted in a downregulation or shutdown of a number of STAT3 downstream targets (Figure 2.1). Cyclin D1 and Bcl-xL levels were gradually downregulated but expression was still detectable at 60 ng/mL of tetracycline treatment. In contrast, Bcl-2 and Mcl-1 levels were completely undetectable at approximately 20 ng/mL of tetracycline.

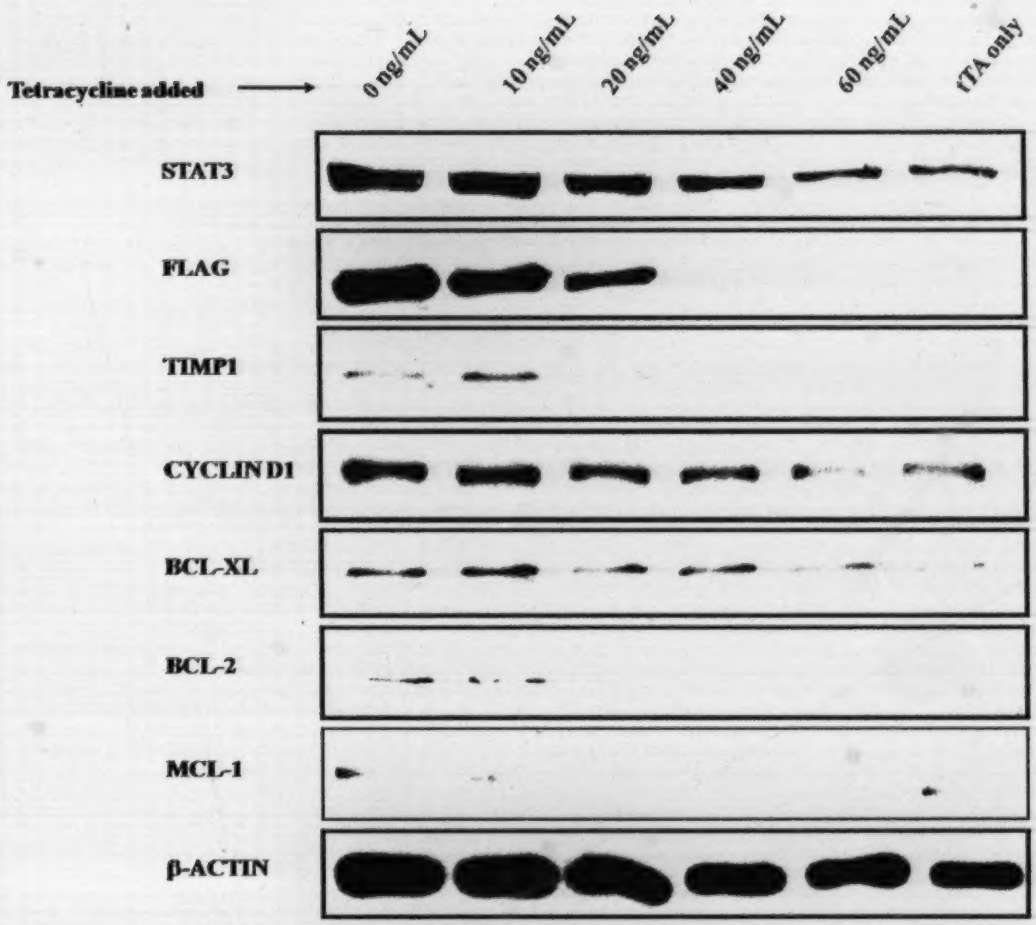


Figure 2.1. Characterization of MCF-7 transfected with tTA-TRE-STAT3C. Western blot analysis of STAT3, FLAG, TIMP1, cyclin D1, Bcl-2, Bcl-xL, Mcl-1 expression in MCF-7 cells transfected with tTA-TRE-STAT3C and treated with increasing concentration of tetracycline for 24 hours. Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. There was a gradual decrease in STAT3 and FLAG with increasing concentrations of tetracycline, with FLAG being completely undetectable at 60 ng/mL. Downregulation of cyclin D1, Bcl-2, Bcl-xL, and TIMP1 expression was identified in the same experiment. MCF-7 cells transfected with tTA alone served as negative controls. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

To show that the link between the expression of STAT3C and TIMP1 is not specific to MCF-7, similar experiments were performed using another breast cancer cell lines, MDA-MB436. After selecting a cell clone permanently transfected with tTA, these cells were then transfected with TRE-STAT3C and treated with various dosages of tetracycline. Figure 2.2 illustrates a gradual downregulation of FLAG and TIMP1 that was dependent on the tetracycline level. At 100 ng/mL, FLAG and TIMP1 were completely undetectable. MDA-MB-436 cells transfected with tTA alone was included as a control and revealed no detectable FLAG or TIMP1 expression.

To further support that STAT3C represents a constitutively activated STAT3 species, immunofluorescence staining and confocal microscopy was employed. In MCF-7 cells permanently transfected with tTA and transiently transfected with TRE-STAT3C (tTA-TRE-STAT3C), STAT3 was localized to the nucleus (Figure 2.3). In contrast, STAT3 was primarily confined to the cytoplasm in MCF-7 cells transfected with tTA only.

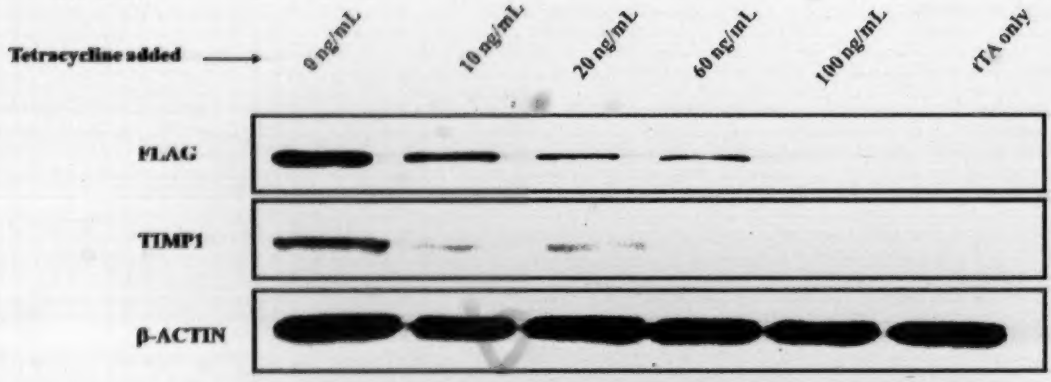
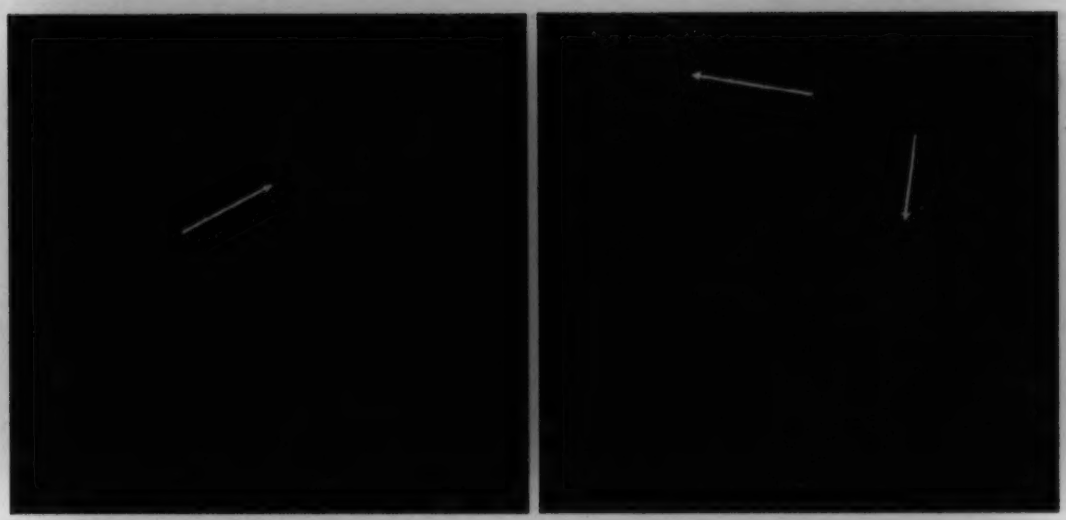


Figure 2.2. Characterization of MDA-MB-436 transfected with tTA-TRE-STAT3C. Western blot analysis of FLAG and TIMP1 expression in MDA-MB-436 cells transfected with tTA-TRE-STAT3C and treated with increasing concentration of tetracycline for 24 hours. Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. A gradual decrease in FLAG and TIMP1 was observed. MDA-MB-436 cells transfected with tTA alone served as negative controls. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.



MCF-7 transfected with tTA only

MCF-7 transfected with tTA-TRE-STAT3C

Figure 2.3. Nuclear localization of STAT3C. Immunofluorescence staining and confocal microscopy was employed to detect STAT3 nuclear localization. MCF-7 cells transfected with either tTA or tTA-TRE-STAT3C were fixed, permeabilized, and stained with Alexa 488 to detect STAT3 expression. Immunofluorescence staining of slides with antibodies against STAT3 and confocal microscopy demonstrated that STAT3 was localized to the nucleus in MCF-7 cells transfected with tTA-TRE- STAT3C, as illustrated by the white arrows (right) whereas STAT3 was confined to the cytoplasm in cells transfected with tTA only (left).

2.3.2. STAT3 transgene correlated with TIMP1 expression

To establish the link between TIMP1 expression and STAT3 signaling in MCF-7 cells, STAT3C expression (as indicated by total STAT3 and FLAG expression) and TIMP1 expression was correlated in tTA-TRE-STAT3C transfected MCF-7 cells using Western blots. As shown in Figure 2.1, STAT3 and FLAG downregulation correlated with a substantial decrease in TIMP1 expression as the tetracycline dosage was increased to 20 ng/mL. To further investigate the role of STAT3 activation in TIMP1 up-regulation, a TIMP1 ELISA assay was also performed in MCF-7 to quantify the TIMP1 levels in the cell culture medium. MCF-7 cells transfected with tTA-TRE-STAT3C and treated with 0 and 60 ng/mL of tetracycline were incubated at 37°C and 5% CO_2 for 72 hours and supernatant from the cell culture medium were collected daily. Figure 2.4 illustrates that TIMP1 levels in MCF-7 cells transfected with tTA-TRE-STAT3 increased in the cell culture over the 3-day period, but the levels for each day were significantly lower in those cells treated with 60 ng/mL of tetracycline. The differences in the TIMP1 levels between the two conditions were not due to a difference in the total cell number in the cell culture, since the cell counts in both conditions were not significantly different from day 1 to day 3. Furthermore, cell cycle analysis using deep red fluorescing anthraquinone Nr.5 (DRAQ5) and flow cytometry showed no significant change in the cell cycle status between these two conditions (data not shown). In addition, MCF-7 cells transfected with tTA alone showed a low levels of TIMP1 (15 ng/mL) at Day 1, and the level was comparable to that found in MCF-7 transfected cells after one day treatment with 60 ng/mL of tetracycline. Similar results were found for Day 2 and 3.

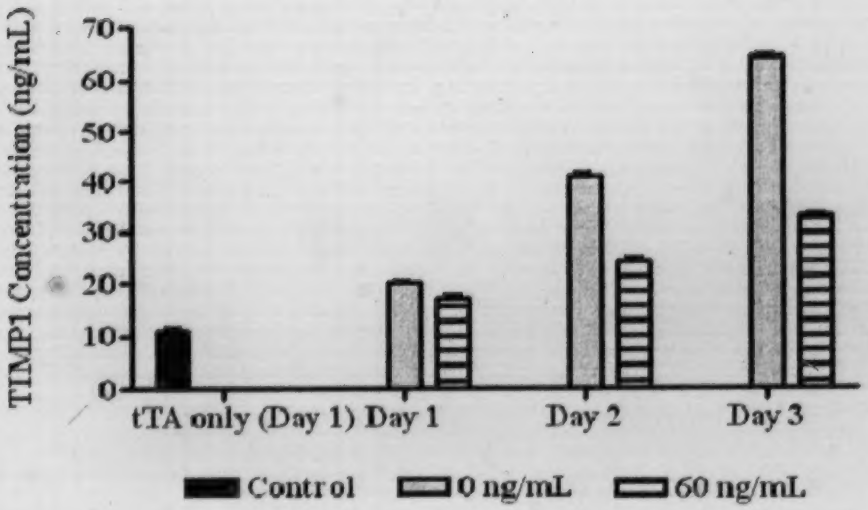


Figure 2.4. STAT3C correlated with TIMP1 level. TIMP1 levels were measured using an ELISA assay in MCF-7 cells transfected with tTA-TRE-STAT3C and treated with 0 and 60 ng/mL of tetracycline. Supernatants from the tissue culture were collected on Day 1, 2, and 3. TIMP1 levels were significantly higher in cells treated with 60 ng/mL than cells with no tetracycline in all 3 days. Increasing levels of TIMP1 (ng/mL) was observed with the duration of experiment, but the rate of increment was significant higher in cells without tetracycline. Day 1 supernatant collected from MCF-7 transfected with tTA alone were included as a reference. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of mean.

2.3.3. Modulation of invasiveness by TIMP1 and STAT3

To investigate the biological significance of STAT3 and TIMP1 in breast cancer, I addressed the question of whether different expression levels of STAT3C and TIMP1 expression would have any effect on the invasiveness of breast cancer cells. An *in vitro* assay for invasiveness using matrigel was performed. As shown in Figure 2.5, invasiveness correlated with the dosages of tetracycline used, and thus, inversely correlated with levels of STAT3C and TIMP1. The assay was also performed on MCF-7 cells prior to transfection with tTA-TRE-STAT3C and an average of 144 cells at 40X were observed, which is similar to the level observed in MCF-7 transfected cells when treated with 60 ng/mL of tetracycline where no FLAG or TIMP1 levels were detected. To determine whether the differences in the cell invasion observed are directly linked to STAT3-regulated TIMP1 expression, a neutralizing anti-TIMP1 antibody was added to MCF-7 cells transfected with tTA-TRE-STAT3C but no tetracycline treatment (highest level of STAT3C). Invasiveness was increased with increasing concentrations of the TIMP1 neutralizing antibody (ranged from 0 to 2.0 μg/mL) (Figure 2.6). The same experiment was repeated using an irrelevant antibody (anti-FLAG) rather than the anti-TIMP1 and no significant changes were detected.

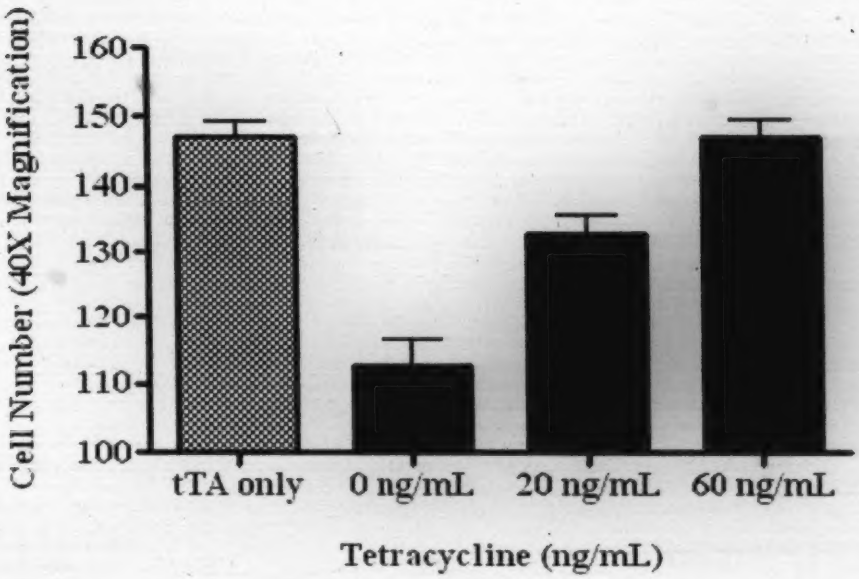


Figure 2.5. STAT3 and TIMP1 levels inversely correlated with invasion. An increase in matrigel invasion was observed in MCF-7 cells transfected with tTA-TRE-STAT3C and treated with increasing levels of tetracycline for a total of 48 hours. Cells treated with tetracycline displayed a higher degree of invasion in a dose-dependent fashion. MCF-7 cells transfected with tTA only were used as controls. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of mean.

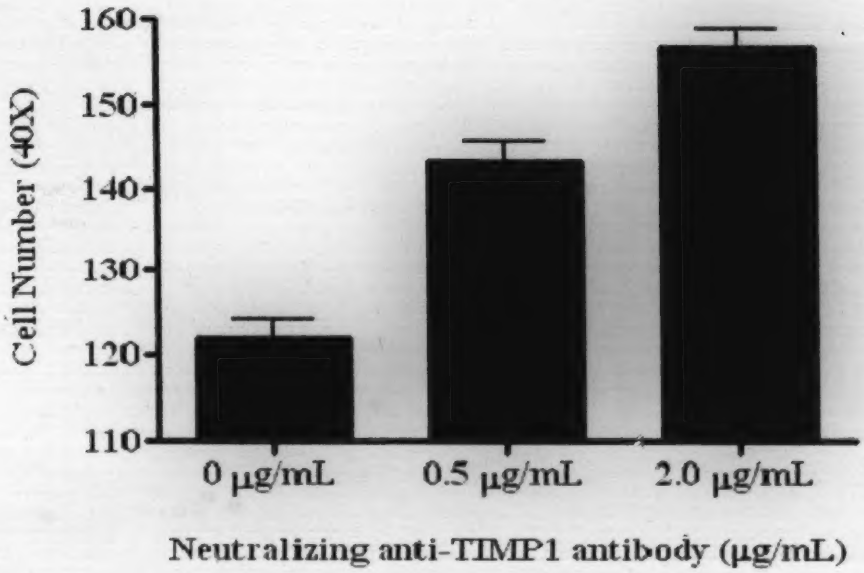


Figure 2.6. TIMP1 directly modulated cell invasion. MCF-7 cells transfected with 'tetracycline-off' STAT3C were treated with 0, 0.5, 2.0 μg/mL of neutralizing anti-TIMP1 antibody. Invasiveness of matrigels was increased in cells with higher levels of neutralizing anti-TIMP1. No tetracycline was added to the cell culture. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of mean.

2.3.4. Correlation between STAT3 activation and TIMP1 expression, lymphatic, and vascular invasion in breast cancer primary tumors

To validate the *in vitro* data, the expression of pSTAT3 and TIMP1 using immunohistochemical analysis was evaluated and compared to lymphatic and vascular invasion. 142 paraffin-embedded, primary breast carcinomas were randomly selected and the clinical characteristics of these 142 cases of breast cancer were summarized in Methods and Materials. Figure 2.7 illustrates the immunohistochemical staining results of breast carcinomas that exhibited positive or negative staining of pSTAT3 and TIMP1. Pictures A and C (left panel) showed a breast cancer case with positive pSTAT3 and TIMP1 staining, whereas pictures C and D (right panel) showed a breast cancer case that was negative for both pSTAT3 and TIMP1. Normal breast sections were generally pSTAT3 negative. The overall results are summarized in Table 2.1. Of the 142 primary breast carcinomas examined, 85 (60%) cases were pSTAT3 positive whereas 112 (79%) were TIMP1 positive. The correlation between pSTAT3 and TIMP1 expression is significant (*P* 0.0041, Chi square test). A significant correlation between pSTAT3 and lymphatic and vascular invasion was also found in breast cancer (*P* values 0.0002, 0.015, respectively, Chi square test). TIMP1 expression also showed a trend for a lack of vascular and lymphatic invasion, although the correlation is not statistically significant (*P* values 0.29, 0.35, respectively, Chi square test).

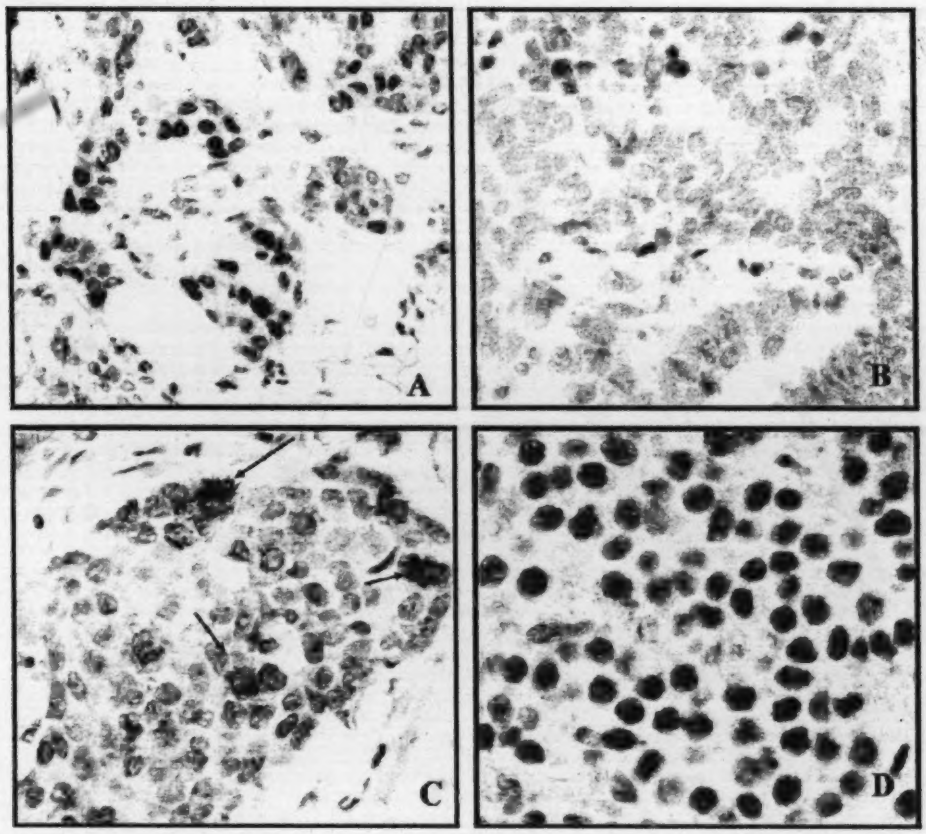


Figure 2.7. pSTAT3 and TIMP1 expression in primary breast tumors. Immunohistochemical staining of paraffin-embedded tissue sections using pSTAT3 (A and B) and TIMP1 (C and D) applied to breast cancer tissues. Strong nuclear pSTAT3 expression as shown in A correlated with strong cytoplasmic TIMP1 expression as shown in C (arrow). Another case was negative for pSTAT3 and TIMP1 (B and D).

Table 2.1. Correlation between STAT3 activation and TIMP1 expression, lymphatic invasion and vascular invasion

	STAT3 Activation		
	Positive	**Negative**	**P values**
TIMP1			
(total cases assessed = 142)			
Positive	75	37	**0.0041**
Negative	10	20	
Lymphatic			
(total cases assessed = 85)			
Positive	12	26	**0.00020**
Negative	34	13	
Vascular			
(total cases assessed = 120)			
Positive	22	29	**0.015**
Negative	46	23	

2.3.5. Correlation between STAT3 activation and TIMP1 expression and other clinical parameters in breast cancer patients

The expression of pSTAT3 and TIMP1, as evaluated by immunohistochemical analysis, were correlated with overall survival of patients at 5 years after the initial diagnosis. Kaplan-Meier survival curves were generated for 55 patients with primary breast cancer stained for pSTAT3 expression and for 68 patients with primary breast cancer stained for TIMP1 expression. Patients with pSTAT3-positive tumors showed a trend for better survival at 5 years (*P* value 0.13, Logrank), although the results are not statistically significant. Similarly, patients with TIMP1-positive tumors also showed a trend for better survival at 5 years (*P* value 0.39, Logrank). Both STAT3 and TIMP1 expression did not significantly correlate with the clinical stage, histologic grade or expression of hormone receptors.

2.4. Discussion

STAT3 is a latent cytoplasmic transcription factor that has been shown to be oncogenic when constitutively activated (Bromberg J and Darnell, 2000; Shuai, 2000). Activation of STAT3 is tightly linked to the development and differentiation of normal breast tissues, with STAT3 activation found during puberty, early pregnancy, and involution, but not detectable in the later parts of pregnancy and lactation (Groner B, 2000; Hennighausen et al., 1997; Philp et al., 1996). The exact biological functions of STAT3 in breast tissues are not fully understood, but it appears to exert pro-apoptotic effect during involution. Immunohistochemical studies of breast cancer tissues have demonstrated evidence of STAT3 activation in the malignant component but not in the

surrounding benign breast tissues (Berclaz G, 2001). Moreover, previous studies have shown that STAT3 activation is detectable in 30 – 60% of primary breast cancers (Berishaj et al., 2007; Dolled-Filhart et al., 2003; Watson and Miller, 1995). Although the mechanism by which STAT3 is activated in breast cancer is not fully understood, it is known that STAT3 is activated in breast cancer cells in response to a diverse number of cytokines and growth factors known to be important for the pathogenesis of breast cancer, such as OSM, EGF and PDGF (Bromberg J, 2000; Darnell JE Jr, 1997; Hirano T, 2000). It is likely that STAT3 is activated via a combination of different mechanisms, since the level of STAT3 activation (i.e. percentage of pSTAT3-positive cells) varies from case to case.

While little is known about the normal function of STAT3 in breast tissues, even less is known about the significance of STAT3 in the pathogenesis of breast cancer. Therefore, the goal in this chapter was to confirm the activation of STAT3 in breast cancer and to further delineate the mechanism by which STAT3 mediates its oncogenic effects in breast cancer. Using immunohistochemistry, I was able to confirm the findings of previous studies and found evidence of STAT3 activation in approximately 60% (85/142) of tumors examined in this current study. In regards to *in vitro* studies, a STAT3C model controlled by tetracycline was employed. The majority of the experiments in this study involved MCF-7, a human breast cancer cell line previously reported to have very low constitutive STAT3 activation (Berishaj et al., 2007). I was able to confirm this finding by immunofluorescence staining/confocal microscopy as well as Western blot using anti-pSTAT3. In the absence of constitutive activation of endogenous STAT3, STAT3

activity was manipulated by transfecting tet-off STAT3C, a construct that have been extensive characterized and published (Bromberg et al., 1999), into the cells. In keeping with the fact that STAT3C represents a species with constitutive activity in our experimental model, immunofluorescense demonstrated that transfected STAT3C was localized to the nucleus whereas endogenous STAT3 was localized to the cytoplasm. Furthermore, to confirm that STAT3C is biologically active, a number of STAT3 downstream targets (including cyclin D1) were up-regulated as a result of tTA-TRE-STAT3C.

Since one of the major goals of this chapter is to evaluate the role of STAT3 in modulating invasion in breast cancer, I sought to examine the link between STAT3 and TIMP1, a low molecular weight glycoprotein that is known to inhibit the proteolytic activity of MMPs (Lambert et al., 2004) to decrease cell migration and invasion (Akahane et al., 2004). Using the established experimental model, I demonstrated that TIMP1 expression correlated well with the expression of STAT3C. This is in keeping with the concept that TIMP1 is a downstream target of STAT3. Other supporting evidences is as follows: 1) the promoter region of the *TIMP1* gene has multiple STAT3 binding sites (Bugno et al., 1995); 2) our lab have previously shown that a STAT3 dominant negative construct can down-regulate TIMP1 expression in lymphoma cell lines constitutively expressing high levels of TIMP1 (Lai et al., 2004); 3) breast cancer cell lines transfected with STAT3C, a well-characterized constitutively active STAT3 construct, have up-regulation of TIMP1; 4) using the created tet-off STAT3C system, I demonstrated that the levels of STAT3 activated significantly correlated with the TIMP1

levels in breast cancer cells; 5) the significant correlation between pSTAT3 and TIMP1 in a cohort of primary breast tumors. There have been a number of recent studies that have further confirmed the link between STAT3 and TIMP1 in oncogenesis. For example, Guo *et al.* demonstrated that IL-6 induction of TIMP1 in hepatocellular carcinoma cells was dependent on STAT3 activation (Guo et al., 2007). Interestingly, a recent study indicated that TIMP1 expression induces STAT3 activation in plasmacytic tumors (Guedez et al., 2005). Taken together, there may be a positive feedback between TIMP1 and STAT3 in certain cancer cell types. Importantly, since TIMP1 plays a major role in cell invasion, the matrigel invasion assay was able to demonstrate that the invasiveness of breast cancer cells was inversely proportional to the expression levels of STAT3C and TIMP1. Using an anti-TIMP1 neutralizing antibody, I have provided direct evidence that TIMP1, rather than some other non-specific factors, directly contributes to the decreased invasiveness in the experimental model. In parallel to these *in vitro* findings, using a large series of primary breast cancer, STAT3 activation highly correlates with TIMP1 expression, and inversely correlates with lymphatic and vascular invasion.

Although the inverse relationship between STAT3 activation and vascular or lymphatic invasion appears to contradict the established pro-oncogenic properties of STAT3, it is of note that TIMP1, a STAT3 downstream target, possesses cell growth-stimulatory functions (Nakopoulou et al., 2003; Visse and Nagase, 2003). TIMP1 has been shown to inhibit apoptosis in several tumor cell types. For instance, Guedez *et al.* showed that TIMP1 directly contributes to increase of survival in Burkitt lymphoma cells (Guedez et

al., 1998). Further studies indicated that TIMP1 can initially promote tumor growth in the early stages of lymphoma by inhibiting apoptosis, whereas at a later stage, the secreted TIMP1 blocks tumor angiogenesis and ultimately suppress tumor growth (Guedez et al., 2001), suggesting that TIMP1 may have a dual role in oncogenesis. More recently, Liu *et al.* has provided evidence that TIMP1 contributes to the survival of breast cancer cells by inhibiting apoptosis (Liu et al., 2005c). In addition, Luparello *et al.* did a comparison study between two breast cancer cell lines and found the more tumorigenic one to be more responsive to TIMP1 treatment, indicating a proliferative role for TIMP1 (Luparello C, 1999). The mechanism by which TIMP1 promotes cell proliferation and inhibits apoptosis has been reported to be either by direct entry into the nucleus or by initiation of the signaling cascades (Ornbjerg Wurtz S, 2005). Thus, while TIMP1 downregulates the invasiveness of breast cancer cells, it provides survival signal and promotes growth of the tumors. In view of this dual function of TIMP1, it is tempting to hypothesize that high levels of TIMP1 may result in large but localized (i.e. non-metastatic) tumors. In other words, differences in the STAT3 activity and TIMP1 expression may determine how early metastasis occurs during the course of the disease. In keeping with this hypothesis, it is well recognized that some breast cancer patients present with metastatic diseases but relatively small primary tumors, and some other patients present as localized disease despite the presence of large primary tumors.

In addition to TIMP1, STAT3 is also known to mediate resistance to apoptosis in breast cancer by up-regulating other proteins known to promote cell survival, such as survivin (Hsieh et al., 2005). Other potential STAT3 downstream targets, including cyclin D1 and

Bcl-2, are also known to play roles in the pathogenesis of breast cancer. In this study, using MCF-7 transfected cells, cyclin D1, Bcl-2, Bcl-xL, and Mcl-1 were examined and a downregulation of STAT3 by increasing concentrations of tetracycline also resulted in a downregulation of these specific targets. Recent studies have speculated that STAT3 signaling may contribute to cell adhesion. Rivat *et al.* stated that the STAT3 signaling pathway regulates homotypic cell-cell adhesion in colorectal cancer cells (Rivat et al., 2004). Another recent publication has suggested a role for STAT3 as a positive regulator of cell-cell adhesion system (Uttamsingh et al., 2003). Overall, STAT3 appears to carry important roles in modulating many biological aspects of breast cancers, including cell survival, proliferation, and metastatic potential.

Increased expression of TIMP1 has been associated with decreased invasiveness and metastatic potential in several experimental models by downregulating MMP activity (Fassina et al., 2000; Gomez et al., 1997; Visse and Nagase, 2003). Interestingly, our lab previously identified high levels of STAT3 activation and TIMP1 expression in a specific type of lymphoma, ALK^+ALCL (Lai et al., 2004). In accordance with the concept that TIMP1 inhibits invasiveness, one of the most characteristic pathologic features of ALK-positive anaplastic large cell lymphomas is their sinusoidal infiltrative pattern within the lymph node. Thus, the link between STAT3 activation and the relative absence of vascular or lymphatic invasion in breast cancer parallels with what we previously observed in ALK^+ALCL.

The fact that a significant proportion of TIMP1-positive breast tumors showed no evidence of STAT3 activation suggests that TIMP1 can be activated via alternative mechanisms in this cell type. A relatively small number of cytokines have been shown to up-regulate TIMP1 in various cell types (Blavier et al., 1999), including IL-6 in human fibroblast (Sato et al., 1990) and IL-10 in human mononuclear phagocytes (Lacraz et al., 1995). TNF receptor (TNFR) has also been shown to stimulate TIMP1 secretion in myeloblastic leukemia cells (Kubota et al., 1996). It will be of interest to evaluate if these cytokines play a role in up-regulating TIMP1 in breast cancer. A subset of breast tumors that are STAT3 active but TIMP1 negative was also identified. One possible explanation for this finding may be related to the fact that the *TIMP1* promoter has the binding sites for transcriptional factors AP-1 and STAT3; it has been suggested that the presence of both of these factors is required for full induction of TIMP1 transcription (Bugno et al., 1995). In addition, we found that TIMP1 expression is dependent on a relatively high level of STAT3 activity, as TIMP1 was dramatically decreased with a reduction of STAT3C to 74% (Figure 1a).

Previous studies have demonstrated that overexpression of TIMP1 prolong survival in transgenic mice model (Blavier et al., 1999). Though the results were not statistically significant, I did find a trend for longer survival in patients with STAT3 activation and TIMP1 expression. The lack of statistical significance in this current study is probably related to the relatively small sample size for survival data analysis. Thus, further studies with increasing sample size must be done in order to further define the relationship of TIMP1 and patient survival. One previous study has correlated STAT3 activation and

survival in 346 patients with breast cancer, and it was found that STAT3 activation is associated with significantly improved survival at both 5 and 20 years (Dolled-Filhart M, 2003). By immunohistochemical analysis, TIMP1 overexpression in breast cancer cells also has been found to be an indicator of favorable prognosis in breast cancer patients (Nakopoulou et al., 2003). Data from this study suggests that the explanation for the longer survival in patients with STAT3 activation and TIMP1 expression is related to the relative infrequent vascular and lymphatic invasion, both of which are known to be independent poor prognostic factors (Subramaniam and Isaacs, 2005).

STAT3 activation inversely correlated with vascular and lymphatic invasion, and although TIMP1 expression showed a trend for lack of vascular and lymphatic invasion, the results were not statistically significant. The reason that TIMP1 expression did not correlate with vascular and lymphatic invasion may be due to the fact that there are several other factors that may contribute to breast cancer invasion and metastasis. A number of molecules such as cytokines, chemokines, and growth factors have been implicated in the metastasis of breast cancer (Fernandis et al., 2004). In particular, chemokine receptor CXCR4 mediates actin polymerization and pseudopodia formation and induces invasive responses in breast cancer cells (Muller et al., 2001) through interaction with its ligand, stromal cell-derived factor-1 (Liang et al., 2005). The ligands for these CXCR4 receptors have been found to be highly expressed in organs that represent important sites of breast cancer metastasis (Fernandis et al., 2004). CXCR4 expression has been shown to be enhanced by HER2 in breast cancer (Li C, 2004), and CXCR4 receptors have been implicated in the activation of the STAT3 pathway in

hematopoietic cell lines (Ahr et al., 2005). Thus, while TIMP1 contributes to modulating the metastatic potential of breast cancer, a number of other factors are also clearly implicated.

In conclusion, I have confirmed that STAT3 is highly activated in human breast cancer and correlates with a decrease in lymphatic and vascular invasion. Moreover, I have demonstrated a novel link between STAT3 and TIMP1 in breast cancer and that the up-regulation of TIMP1 by STAT3 decreases the invasive abilities of breast cancer cell lines. These findings provide us with a better understanding of STAT3 signaling in breast cancer pathogenesis. In addition, the tet-off STAT3C *in vitro* model created will provide me with a tool for future examination of STAT3 in cancer.

2.5. References

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Chapter 3 ♦

Aberrant Expression of IL-22 Receptor 1 (IL-22R1) and Autocrine IL-22 Stimulation Contribute to Tumorigenicity in ALK-positive Anaplastic Large Cell Lymphoma

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3.1. Introduction

ALK^+ALCL is a specific type of non-Hodgkin lymphoma, characterized by a sinusoidal infiltrative pattern, T/null-cell immunophenotype, and the consistent expression of CD30. The aberrant expression of ALK in approximately 80% of ALK^+ALCL tumors is a result of the reciprocal chromosomal translocation, t(2;5)(p23;q35), which leads to the juxtaposition of the *NPM* gene at *5q35* with the *ALK* gene at *2p23* (Morris et al., 1994; Shiota et al., 1994b). NPM-ALK has been shown to be oncogenic, and it directly contributes to the pathogenesis of ALK^+ALCL through exerting its constitutively active tyrosine kinase embedded in the ALK portion of the fusion protein (Bischof et al., 1997; Duyster et al., 2001; Fujimoto et al., 1996). Previous studies have demonstrated dysregulation of multiple signaling pathways (Slupianek et al., 2001), including that of STAT3 (Khoury JD, 2003; Zamo et al., 2002). STAT3 itself is an oncoprotein, and aberrant activation of STAT3 has been demonstrated in a variety of human cancers (Bromberg et al., 1999; Burke et al., 2001; Catlett-Falcone R, 1999; Levy and Lee, 2002). Moreover, accumulating evidence has supported the concept that NPM-ALK mediates its oncogenic effects via STAT3 activation (Chiarle et al., 2005; Khoury JD, 2003; Zamo et al., 2002; Zhang et al., 2002). Sustained STAT3 activation appears to be multi-factorial in ALK^+ALCL; in addition to NPM-ALK, other mechanisms include Src, JAK3, and loss of SHP1 (Amin et al., 2003b; Fujitani et al., 1997; Han et al., 2006). JAK3, one of the normal physiologic activators of STAT3, is indeed highly activated in most cases of ALK^+ALCL (Lai et al., 2005), and our recent data suggest that the mechanism is related

to the autocrine stimulation of IL-9 and loss of SHP1 (a tyrosine phosphatase) (Han et al., 2006; Qiu et al., 2006).

IL-22 is a cytokine that was originally labeled IL-10-related T-cell-derived inducible factor (Dumoutier et al., 2000). IL-22 belongs to a family of IL-10-related proteins that includes IL-19, IL-20, IL-24/MDA-7, IL-26/AK155, IL-28 and IL-29. IL-22 production is inducible by IL-9 in T-lymphocytes (Dumoutier et al., 2000) and is known to exert its function by binding to a heterodimeric receptor complex composed of IL-22R1 and IL-10R2. IL-10R2 is ubiquitously expressed, while IL-22R1 expression is relatively restricted, being found at a high level in the pancreas, small intestine, colon, kidney, and liver (Kotenko et al., 2001; Xie et al., 2000). Importantly, IL-22R1 is not detectable in normal immune cells including monocytes, B-cells, T-cells, natural killer cells, macrophages and dendritic cells, that are found in the bone marrow, peripheral blood, thymus and spleen (Wolk et al., 2002; Wolk et al., 2005; Wolk et al., 2006). In addition to the IL-10R2 and IL-22R1, there is a single-chain IL-22 binding receptor named IL-22 binding protein (commonly labeled as IL-22BP), which has been shown to be a decoy receptor that can antagonize IL-22 binding to its receptor and downregulate IL-22 signaling *in vitro* (Dumoutier et al., 2001; Gruenberg et al., 2001; Kotenko et al., 2001). IL-22BP is detectable in placenta, breast, thymus, spleen, lymph nodes, stomach and colon (Weiss et al., 2004; Xu et al., 2001). IL-22 is known to activate a number of signaling pathways including that of STAT3 and mitogen-activated protein kinase (MAPK) (Lejeune et al., 2002). Based on the current understanding of the biology of IL-

22, it is believed that IL-22 produced by T-cells homing to the mucosal surfaces plays an important role in enhancing innate immunity (Gurney, 2004).

An initial study in our lab analyzed cytokine expression in ALK^+ALCL cells using oligonucleotide microarrays, and identified the expression of IL-22R1 as well as IL-22. A recent published study using large-scale microarray gene expression profiling also described the expression of IL-22 among ALK^+ALCL tumors and cell lines (Lamant et al., 2006). While the expression of IL-22 is not unexpected for ALK^+ALCL, a tumor derived from mature T-cells, IL-22R1 expression in ALK^+ALCL cells, a type of lymphoid cells, represents an aberrant event, since this receptor is not detectable in normal lymphoid tissues. In view of these findings and the importance of STAT3 signaling in ALK^+ALCL, I hypothesized that the aberrant expression of IL-22R1 creates an abnormal cytokine stimulatory pathway to contribute to the tumorigenicity of ALK^+ALCL. In this study, I first confirmed that IL-22, IL-22R1 and IL-10R2 are consistently expressed in ALK^+ALCL cell lines and tumors, whereas IL-22BP is often absent. Blockade of IL-22 using an IL-22 neutralizing antibody or recombinant IL-22BP decreases STAT3 phosphorylation and activation, which were associated with a decrease in cell proliferation and tumorigenicity. Furthermore, gene transfection of *NPM-ALK* demonstrated its role in inducing aberrant expression of IL-22R1 in a T-cell lymphoma cell line.

3.2. Materials and Methods

3.2.1. Cell lines and Culture

Three ALK^+ALCL cell lines, SU-DHL-1, Karpas 299, and SUP-M2 were used in this study. All three cell lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). Hepatocellular carcinoma cell line HepG2 (ATCC, HB-8065) and T-lymphoblast cell line Jurkat (ATCC, TIB-152) was also used in this study. ALCL cell lines were maintained in RPMI-1640 (Sigma-Aldrich). HepG2 was maintained in DMEM (Sigma-Aldrich). Both types of culture media were enriched with 10% FBS (Gibco) and antibiotics (10,000 units/mL penicillin G, 10,000 µg/mL streptomycin, Gibco). All cells were grown at 37°C in 5% CO_2. T- and B-lymphocytes were harvested from peripheral blood of a healthy donor cultured in RPMI-1640. Briefly, lymphocytes were isolated using Ficoll-Paque Plus according to the manufacturer's protocol (GE Healthcare Biosciences, Uppsala, Sweden). CD3-positive T-cells and CD19-positive B-cells were then isolated using the Epics Altra Hypersort system (Beckman Coulter, Mississauga, Ontario, Canada).

3.2.2. Treatment of Cells

For cytokine experiment, cells (1 X 10^6 cells/mL) were washed twice with PBS and plated into 6-well culture plates (Bectin Dickinson Labware) with serum-free media for 16 hours. Cells were treated with 5 µg/mL of IL-22BP recombinant protein (R&D Systems, Minneapolis, MN, USA) or 10 µg/mL of IL-22 neutralizing antibody (Sigma) for 0, 30 min, 1 hour, 4 hour, and 24 hour. In addition, cells were also treated with 10

ng/mL of IL-22 recombinant protein (Sigma) for 0 and 30 min. All cell samples were harvested for western blot.

3.2.3. Western Blot Analysis and Antibodies

Western blot analysis was performed using standard techniques. Briefly, cells were collected, centrifuged at 300*g* for 10 minutes, and washed twice with PBS. Lysis buffer (20 mM tris-HCl, pH 7.5, 5.0 mM EDTA, 40.0 μg/mL leupeptin, 1 μM pepstatin, 1 mM AEBSF) was added to the cell pellet for 30 minutes on ice followed by centrifugation at 14,000*g* for 10 minute at 4°C. The supernatant was removed and 50-100 μg of protein was run on SDS-PAGE. Protein quantification was carried out using the Bradford assay according to the manufacturer's protocol (Biorad). After the proteins were transferred to nitrocellulose membranes, the membranes were blocked with 5% milk in TBS buffer (Sigma) and 0.05% Tween-20 (Calbiochem), and then incubated with primary antibodies overnight followed by 1 hour incubation with horseradish peroxidase-conjugated secondary antibody (1:10000) (Jackson Immunoresearch Laboratories). The membranes were washed in TBS with 0.05% Tween-20 for 30 minutes between steps. Proteins were detected using the enhanced chemiluminescence detection kit (Amersham Life Sciences). The number in parenthesis after each antibody listed indicates the dilution at which it was used. Antibodies used included: rabbit polyclonal anti-human STAT3 (1:500, Santa Cruz), mouse monoclonal anti-human pSTAT3 (1:500, Santa Cruz), rabbit polyclonal anti-human IL-22R1 (1:1000, Sigma-Aldrich), rabbit monoclonal anti-human pERK1/2 (1:1000, Cell Signaling), mouse monoclonal anti-human ERK1/2 (1:1000, Cell Signaling), rabbit monoclonal anti-human anti-pJNK/SAPK (1:1000, Cell Signaling),

rabbit monoclonal anti-human JNK/SAPK (1:1000, cell Signaling), rabbit monoclonal anti-human phospho-p38 (1:1000, Cell Signaling), mouse monoclonal anti-human p38 (1:1000, Cell Signaling) and mouse monoclonal anti-human β-actin (1:3000, Sigma-Aldrich).

3.2.4. Immunofluorescence Staining and Confocal Microscopy

Immunofluorescence was performed using standard techniques. Briefly, 1 X 10^6 cells cells grown on coverslip in a 6-well plate were fixed with 4% paraformaldehyde in PBS. Cells were rinsed with 1X PBS, permeabilized with PBS-0.5% triton X-100 for 5 minutes, and rinsed twice with 1X PBS. Cells were rinsed three times with 1X PBS, incubated with 30 µL of anti-IL-22R1 (1:50) (Sigma-aldrich) antibody overnight, followed by rinsing three times with 1X PBS. IgG antibody was included as a negative control. After Incubation with 25 µL of Alexa 488 goat anti-rabbit secondary antibody (1:250) for 1 hour, cells were rinsed with PBS and mounting media (Sigma-aldrich) was added to the slides. Cells were visualized and imaged with a Zeiss LSM 510 confocal microscope (Oberkochen, Germany). Argon laser with a 488 nm wavelength was used to visualize IL-22R1 at 40X objective and images were analyzed using the Zeiss LSM 5 image browser.

For the double staining experiments, frozen sections from 5 cases of ALK^+ALCL were fixed in acetone and washed with PBS. Slides were incubated simultaneously with anti-IL-22R1 (Capralogic, 1:200, anti-goat) and anti-IL-22 (Capralogic, 1:200, anti-rabbit) for 1 hour. After washings with PBS, slides were incubated simultaneously with Alexa 488

goat anti-rabbit and Alexa 555 donkey anti-goat secondary antibodies (1:300) for 30 minutes at room temperature. Mounting media with DAPI were added to the slides and were visualized with a Zeiss LSM 510 confocal microscope (Oberkochen, Germany). Argon laser with a 488 nm wavelength was used to visualize IL-22R1 and Helium/Neon laser with a 623 nm wavelength was used to visualize IL-22. All images were visualized at 40X objective and analyzed using the Zeiss LSM 5 image browser.

3.2.5. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted from SU-DHL-1, Karpas 299, SUP-M2, Jurkat, and HepG2 cell lines using TRIzol extraction according to manufacturer's protocol (Gibco). Reverse transcription (RT) was performed using 500 ng total RNA in a first strand cDNA synthesis reaction with superscript reverse transcriptase as recommended by the manufacturer (Invitrogen). Primer pairs were designed by Primer3 Input 0.4.0 to detect IL-22, IL-22R1, IL-22BP, IL-10R2 and NPM-ALK. Housekeeping genes, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-globin were included as internal controls. Table 3.1 illustrates the primer sequence and product size for each gene. Polymerase chain reaction (PCR) was performed by adding 1 μL RT product into 25 μL volume reaction containing 1X buffer, 200 μM of each dNTPs, oligonucleotide primer, and 0.2 U Ampli*Taq* polymerase. For DNA amplification, cDNA was denatured at 94°C for 1 min, and then subjected to primer annealing at 60°C for 1 min, and then subjected to DNA extension at 72 °C for 1 min for 35 cycles in a thermal cycler (Applied Biosystems). Amplified product were analyzed by DNA gel electrophoresis in 1% agarose and visualized by the Alpha Imager 3400 (Alpha Innotech, San Leandro, CA,

USA). Sequencing of IL-22 PCR product was performed using TOPO TA Cloning Kit according to manufacturer's protocol (Invitrogen) and the 3130XL Genetics Analyzer (Applied Biosystems).

Table 3.1. Primer Sequences and Product Size

Sense Primers		Antisense Primers	Product size (bp)
IL-22	TTCTCTTGGCCCTCTTGGTA	TTCTCCCCAATGAGACGAAC	169
IL-22R1	TGCTGACCATCTTGACTGTG	TCCCTCTCTCCGTACGTCTTAT	181
IL-22BP	GGAACTCAGTCAACGCATGA	TTGGCTTCTGGTGAGAGCTT	178
IL-10R2	TACCACCTCCCGAAAATGTC	CAAATTCAGCCCTGACTCTCA	219
GAPDH	AAGGTCATCCCTGAGCTGAA	CCCTGTTGCTGTAGCCAAAT	316
β-GLOBIN	CAACTTCATCCACGTTCACC	GAAGAGCCAAGGACAGGTAC	343

3.2.6. IL-22R1 siRNA

SU-DHL-1 cells (1 X 10^6 cells in 1 mL of culture medium) were transfected with 100 pmol of IL-22R1 or scrambled siRNA (Qiagen) using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The IL-22R1 target sequence is: 5'-CAGGA GCTCCACAGCGGCATA-3'. Cells were harvested 24 hours after the transfection and subjected to Western blot analysis.

3.2.7. Gene Transfection of NPM-ALK

The NPM-ALK cDNA was initially amplified using an *NPM-ALK* plasmid (a gift by Dr. Morris) using a primer set: ALK (forward) 5′-ATGGAAGATTCGATGGACATG-3′ and NPM (reverse) 5′-TCAGGGCCCAGGCTGGTT-3′. The NPM-ALK cDNA was blunt ligated with pCruz His™, which was initially digested with EcoRV. The resulted vector was transformed into Escherichia coli on ampicillin-resistant plates, and plasmids were isolated by a Mini-prep kit (Qiagen). The sequence and orientation of the insert were confirmed by DNA sequencing. Transfection experiments were carried out in 12-well culture plates (Bectin Dickinson Labware) by electroporation technology using Nucleofector Kit V according to the manufacturer's protocol (Amaxa, Cologne, Germany). Briefly, 1 X 10^6 cells were washed with PBS twice and resuspended in 100 µL of Cell Line Nucleofector Solution V and 1 µg of DNA. The cell suspension is transfer to the amaxa certified cuvette and transfected using program A-17 on the Nucleofector I machine (Amaxa). 500 µL of pre-warmed RPMI 1640 is added to the cuvette and the cell suspension is transferred to 12-well culture plate (Bectin Dickinson Labware).

3.2.8. ELISA Assay for IL-22

Aliquots of medium were extracted from SU-DHL-1, Karpas 299, and SUP-M2 cells, spun at 15,000*g*, and supernatant was collected. Assay was performed as per company protocol (R&D systems). Briefly, 100 µL of Assay Diluent and sample were added to the 96 well microplate, covered, and incubated for 2 hours at room temperature. Wells were washed 4X with wash buffer and 200 µL of IL-22 Conjugate was added, followed by 2 hour incubation at room temperature. Wash step is repeated and 200 µL of Substrate solution is then added to each well and incubated in the dark for 30 minutes. 50 µL of Stop Solution is added to read well and a colorimetric plate reader (Biorad) measured each absorbance at 450/570 nm.

3.2.9. Cell Growth Assays

To determine cell viability in cells treated with IL-22 recombinant protein, IL-22BP, and IL-22 neutralizing antibody, daily cells counts along with the MTS assay was performed. For cell count, 25,000 cells were plated in 24-well culture plates with 5% FBS. Recombinant IL-22, IL-22BP, and IL-22 neutralizing antibody were added daily and cells were counted daily using tryphan blue exclusion assay (Gibco). For the MTS assay (Promega, Madison, WI, USA), 5,000 cells were seeded in 96-well culture plates and treated daily with recombinant IL-22, IL-22BP, and IL-22 neutralizing antibody for 3 days. Cell viability was then measured colorimetrically at 450nm using a Microplate reader (Biorad) and absorbance values were normalized using the Microplate Manager 5.2.1 (Biorad).

3.2.10. Colony Formation in Soft Agar

To make the base layer, 1.2% soft agar was prepared by autoclaving Bacto agar (Difco, Detroit, MI, USA) in distilled water. RPMI 1640 is then added to make a 0.6% agar and 3.0 mL of agar is added to the bottle of a 6-well plate and allowed to solidify. Cells were suspended in media containing 0.3% agar and RPMI 1640. 2 mL containing 20,000 cells were plated in a six-well plate coated with the base layer. The cells were fed every 4 days by adding 500 µL of RPMI 1640/10% FBS. Colonies were stained with 0.05% crystal violet at 2 weeks. Quantification of cell colonies was done using at 40X magnification and images of the agar were analyzed using the Olympus 1X70 microscope.

3.2.11. ALK^+ALCL Tumors and Immunohistochemistry

10 paraffin-embedded lymph node biopsies from patients with ALK^+ALCL were obtained from the Cross Cancer Institute (Edmonton, AB, Canada) following approval by the institutional ethics committee. The diagnosis of these cases was based on the criteria established by the World Health Organization Classification scheme, and all cases were confirmed to express ALK by immunohistochemistry. Immunohistochemical staining was performed using standard techniques. Briefly, formalin-fixed, paraffin-embedded tissue sections of 4 µM thickness were deparafinized and hydrated. Heat-induced epitope retrieval was performed using Tris buffer (pH 9.9; Dako) and a RHS. After incubation at 100°C for 10 minutes, slides were washed in running tap water for 5 minutes, and endogenous peroxidase were blocked using 10% H_2O_2 and methanol, followed by 5 minutes wash in running tap

water. Tissue sections were then incubated with goat anti-human IL-22R1 (1:500) (Capralogics, Hardwick, MA, USA) or rabbit anti-human IL-22 antibody (1:500) (Capralogics) overnight in a humidified chamber at 4°C. Both antibodies are specific to peptide antigens and antigen sequence analysis indicates peptide antigen specificity. After 3 wash with PBS, tissue sections were incubated with secondary antibodies: anti-rabbit IgG or anti-goat IgG (Dako) for 30 minutes at room temperature. The tissue sections were incubated with 3,3'-diaminobenzidine/H_2O_2 (Dako) for color development, using hematoxylin as a counterstain. To optimize the IHC staining, benign tonsils were used as positive and negative controls for IL-22 in which the mantle zone B-cells served as negative control. For IL-22R1, liver tissues were used as a positive control and benign tonsils were used as a negative control.

3.2.12. Statistical Analysis

Graphpad Instat 3 software was used for statistical analysis of the data. The association between IL-22 and cell growth was evaluated using the Student's t-test. A P value <0.05 was considered statistically significant. Each assay was performed at least in triplicate and the illustrative graphs are representative of the mean of the triplicate experiments. Error bars represent SEM.

3.3. Results

3.3.1. Expression of IL-22R1, IL-10R2, and IL-22 in ALK$^+$ALCL cell lines and tumors

The expression of IL-22R1 and IL-10R2 in 3 ALK$^+$ALCL cell lines was assessed. As shown in Figure 3.1, RT-PCR was performed and the amplifiable products for IL-22R1 and IL-10R2 were detectable in all 3 ALK$^+$ALCL cell lines (lane a-c). While the IL-10R2 bands were of relatively even intensity among these 3 cell lines, SUP-M2 showed the strongest intensity for IL-22R1. HepG2 and a case of breast cancer served as the positive controls for IL-22R1 (lane d and e). IL-22BP, the naturally occurring IL-22R1 decoy, was not detected in any of the ALK$^+$ALCL cell lines, but expressed in a case of breast cancer (lane e).

Next, IL-22R1 protein expression of IL-22R1 was assessed in ALK$^+$ALCL cell lines using Western blot analysis. As shown in Figure 3.2, IL-22R1 protein was detectable in all ALK$^+$ALCL cell lines (lane a-c) but not in normal peripheral blood T- and B-lymphocytes (lane d-e). Correlating with the results obtained from the RT-PCR assays, IL-22R1 protein was detectable in all ALK$^+$ALCL cell lines, but not in benign T- and B-cells harvested from the peripheral blood of a healthy individual. HepG2, the positive control, again showed strong expression of IL-22R1. To determine the sub-cellular localization of IL-22R1, immunofluorescence staining and confocal microscopy was performed. As shown in Figure 3.3, strong IL-22R1 was detected on the cell membrane and in the cytoplasm of SU-DHL-1, Karpas 299 and SUP-M2 cells.

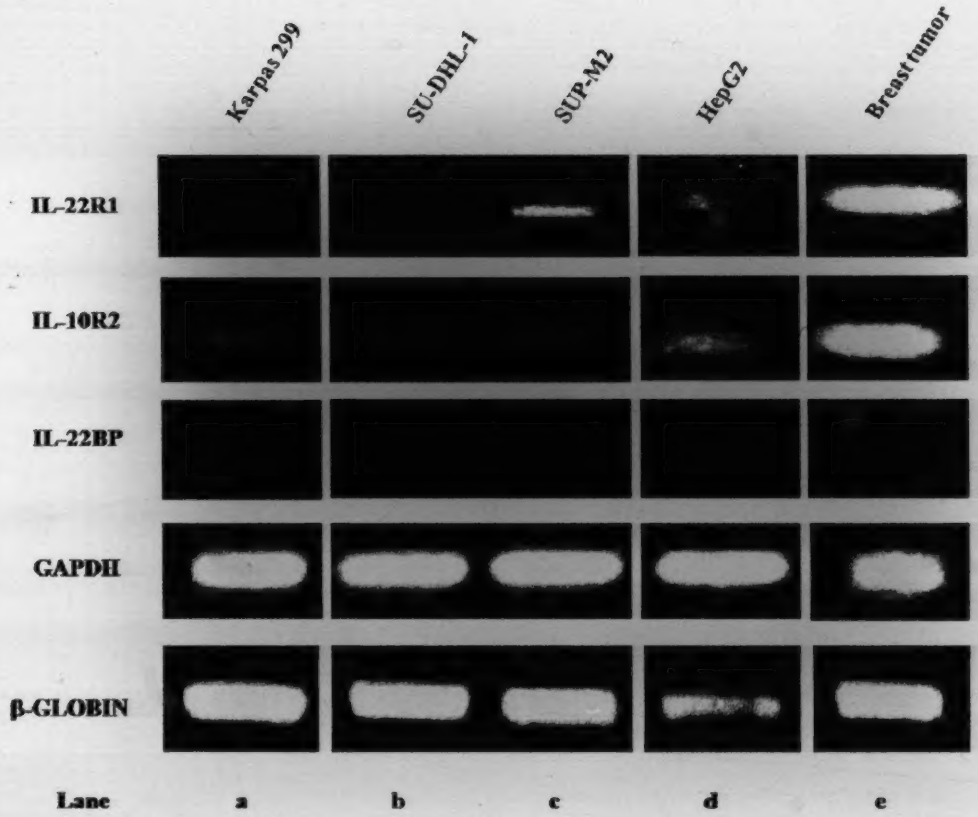

Figure 3.1. mRNA expression of IL-22 receptors in ALK$^+$ALCL. RNA extraction was performed on SU-DHL-1, Karpas 299, SUP-M2 cells, and hepatocellular carcinoma cell lines HepG2. RT-PCR studies demonstrated the presence of IL-22R1 and IL-10R2 mRNA and the absence of IL-22BP mRNA in 3 ALK$^+$ALCL cell lines. HepG2 was included as a positive control for IL-22R1 and IL-10R2 and a breast tumor was included as a positive control for IL-22R1 and IL-22BP. GAPDH and β-globin were included as internal controls.

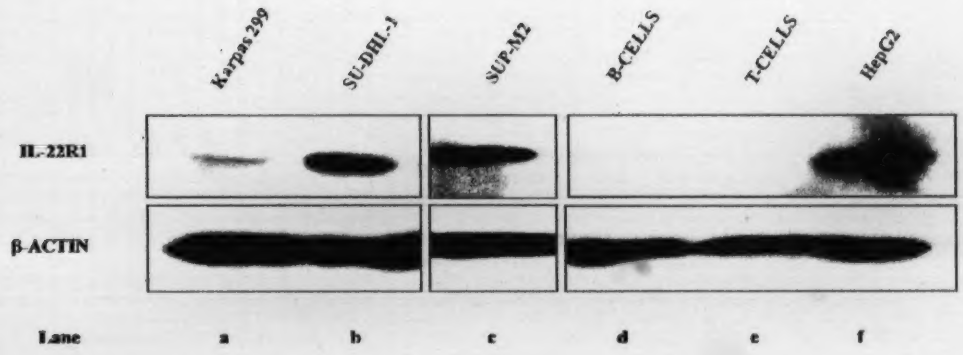

Figure 3.2. Protein expression of IL-22 receptors in ALK$^+$ALCL. Protein lysates for SU-DHL-1, Karpas 299, SUP-M2, HepG2, and T/B-lymphocytes were used and lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. IL-22R1 was detected using an anti-IL-22R1 antibody. Western blot analysis confirmed the expression of IL-22R1 in all 3 ALK$^+$ALCL cell lines examined. T- and B-cells harvested from the peripheral blood of a healthy individual (cell sorted using flow cytometry) were included to confirm the lack of IL-22R1 expression in these cells. HepG2 was included as a positive control. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

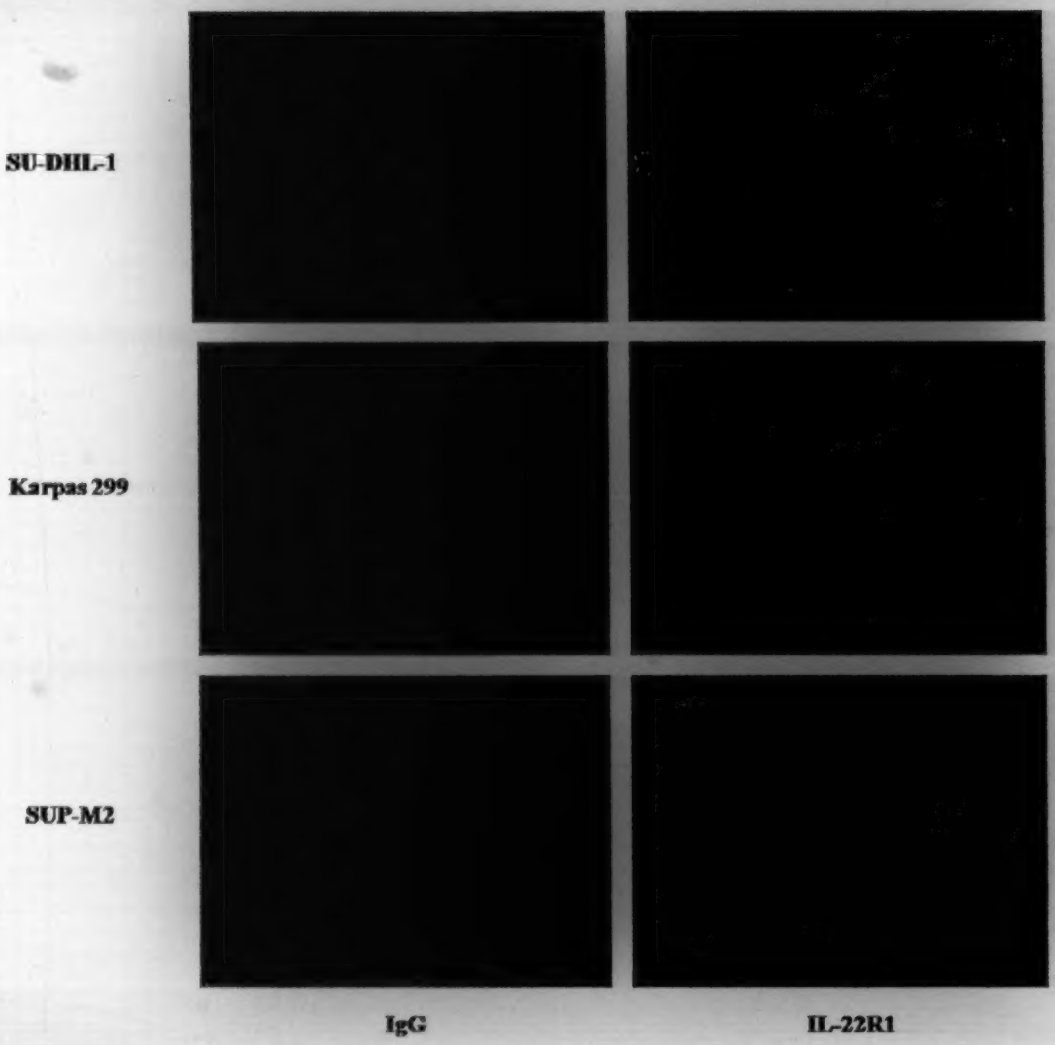

Figure 3.3. IL-22R1 expression in in ALK$^+$ALCL cell lines. Immunofluorescence staining and confocal microscropy was employed to detect IL-22R1 expression on ALK$^+$ALCL cell lines, SU-DHL-1, Karpas 299, and SUP-M2. ALK$^+$ALCL cell lines were fixed, permeabilized, and stained with Alexa 488 to detect IL-22R1 expression. Immunofluorescence staining of slides with antibodies directed against IL-22R1 (left panel) or control IgG (right panel) and confocal microscopy demonstrated the expression of IL-22R1 in ALK+ cell lines, SU-DHL-1, Karpas 299, and SUP-M2. Images were analyzed at 40X objective.

I next sought to determine whether ALK^+ALCL cells express IL-22. As shown in Figure 3.4a, RT-PCR revealed that IL-22 mRNA was expressed in all three ALK^+ALCL cell lines examined. The amplifiable PCR product was sequenced and confirmed to be full-length IL-22 (data not shown). In addition, ELISA assay for human IL-22 was performed using the supernatants of these 3 cell lines; soluble IL-22 was detectable in all three ALK^+ALCL cell lines with the highest levels found in Karpas 299 (Figure 3.4b). HepG2 was included as a negative control since it has been previously reported to have undetectable levels of IL-22 (Radaeva et al., 2004).

To evaluate the expression of IL-22R1 and IL-22, 10 paraffin-embedded tumors and immunohistochemistry was employed. IL-22 was expressed in all tumors examined, as illustrated in Figure 3.5 (upper panel, A and B). Of note, a residual benign B-cell follicle included was not reactive with the anti-IL-22 antibody, thus keeping with the concept that IL-22 is a T-cell derived cytokine and thus not expressed in B-cells. In the lower panel of Figure 3.5 (C and D), scattered ALK^+ALCL cells were reactive with anti-IL-22R1. Consistent with the results from confocal microscopy, IL-22R1 had a membranous/cytoplasmic staining pattern. To further support that IL-22R1 and IL-22 were expressed in the same cells, we employed double immunofluorescence staining and confocal microscopy, and the results are illustrated in Figure 3.6. IL-22 (red signal) and IL-22R1 (green signal) were detected in the same tumor cell.

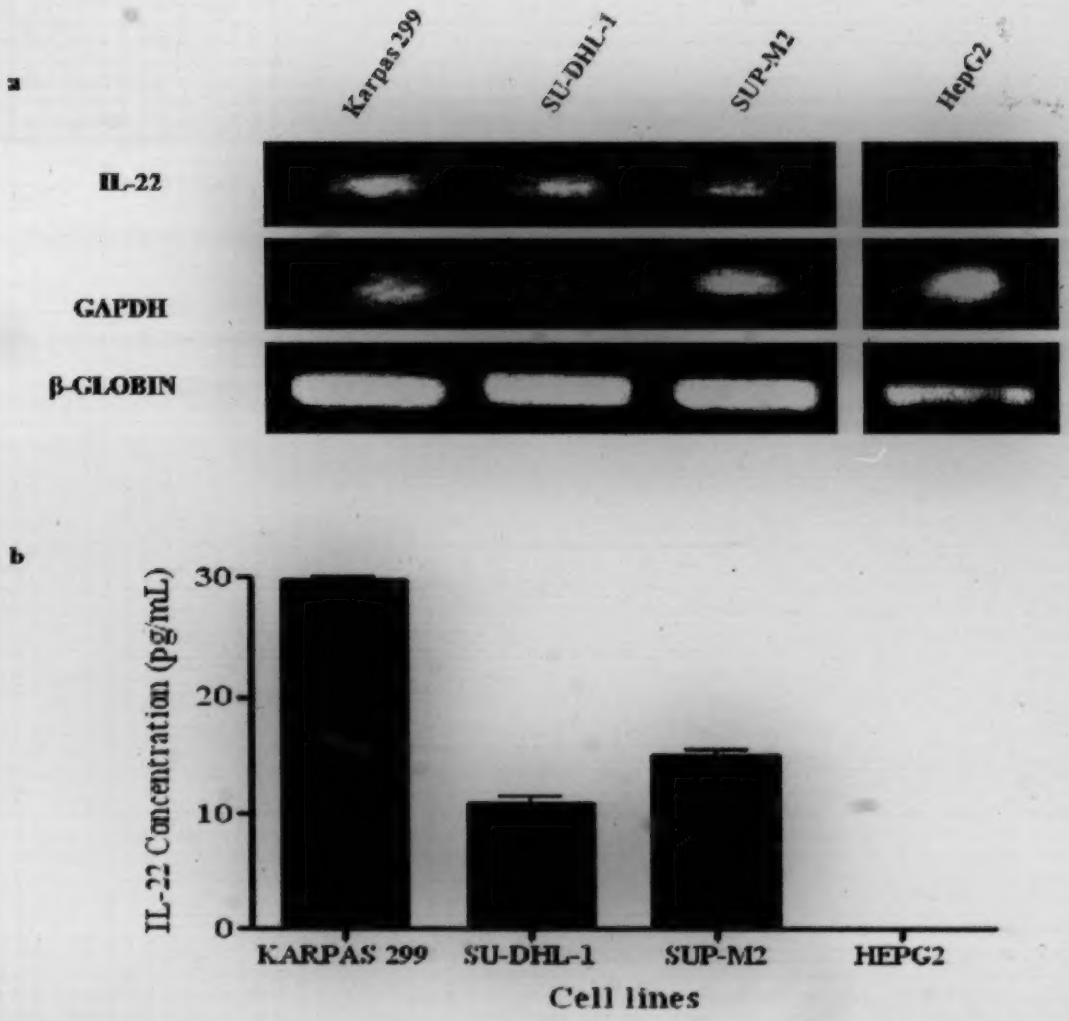

Figure 3.4. Autocrine expression of IL-22 in ALK$^+$ALCL cell lines. (a). RNA extraction was performed on SU-DHL-1, Karpas 299, SUP-M2, and HepG2 cells. RT-PCR studies demonstrated the presence of IL-22 mRNA in ALK$^+$ALCL cell lines. HepG2 cells were included as a negative control and GAPDH was included as an internal control. (b). Supernatant of cell lines were collected and IL-22 ELISA assay was performed to confirm the autocrine expression of IL-22 in ALK$^+$ALCL cell lines. HepG2 was included as a negative control.

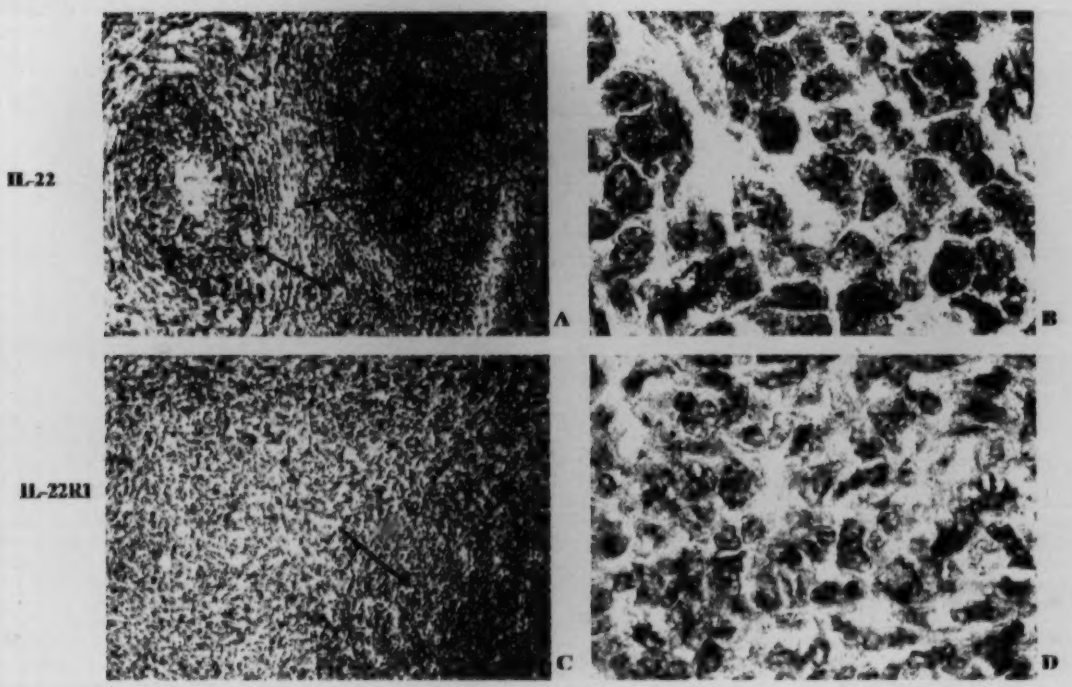

Figure 3.5. IL-22 and IL-22R1 expression in ALK$^+$ALCL tumors. Immunohistochemical staining of paraffin-embedded tissue sections confirmed the expression of IL-22 (upper panel, A and B) and IL-22R1 (lower panel, C and D) in a case of ALK$^+$ALCL tumors. In the upper panel, the red arrow highlights the sinusoid infiltrated by IL-22-expressing ALK$^+$ALCL lymphoma cells, whereas the black arrow highlights a residual lymphoid follicle, which was composed of mostly benign B-cells that were IL-22 negative. B shows a high magnification of the lymphoma cells expressing IL-22. In the lower panel, the infiltrating lymphoma cells were positive for IL-22R1 whereas the residual lymphoid follicle (black arrow) was negative. D shows a high magnification view of the lymphoma cells expressing IL-22R1.

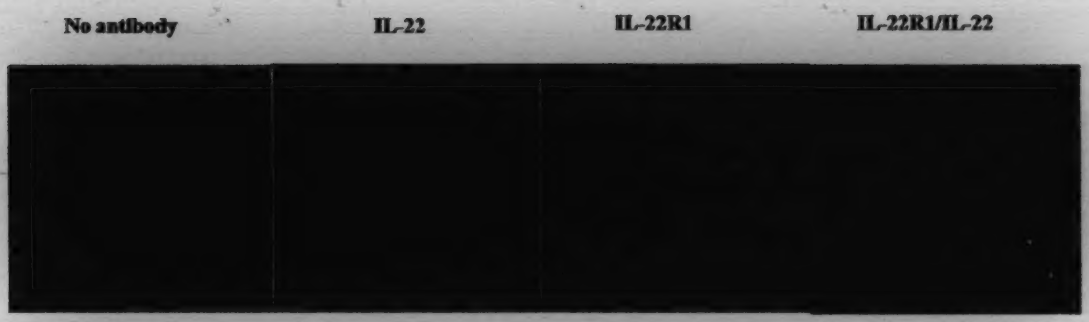

Figure 3.6. Double staining of ALK$^+$ALCL tumor with IL-22 and IL-22R1. Immunofluorescence staining and confocal microscropy was employed to detect double staining of IL-22R1 and IL-22 expression on an ALK$^+$ALCL tumor. ALK$^+$ALCL tumor deparaffinized and stained with Alexa 488 and Alexa 555 to detect IL-22R1 and IL-22 expression. Immunofluorescence staining of slides with antibodies directed against IL-22 (second panel), IL-22R1 (third panel) or no antibody control (first panel) and confocal microscopy demonstrated the expression of IL-22R1 and IL-22. Cytoplasmic/membranous expression of IL-22R1 (red) and IL-22 (green) was demonstrated on the same tumor cell (left image). Images were analyzed at 40X objective.

3.3.2. IL-22 modulates cell growth in ALK^+ALCL

To assess the biological effects of IL-22 stimulation, all three ALK^+ALCL cell lines were serum-starved for 16 hours followed by daily treatment (5 days total) with 10 ng/mL of recombinant IL-22 protein. Serum starvation of cells are required to equilibrate cells to G_0 phase (Gustincich and Schneider, 1993). Daily manual counts of viable cells were performed using trypan blue exclusion. Jurkat cells, a T-lymphoblast cell line that lacks IL-22R1 expression and normal peripheral blood lymphocytes, were included as negative controls. As shown in Figure 3.7, a significant increase in the cell number was observed in all three ALK^+ALCL cell lines on day 3. In contrast, no change in cell growth was observed in Jurkat and normal lymphocytes. To assess the rate of cellular growth, MTS assay was performed on SU-DHL-1 and Karpas 299 cell after treatment of recombinant IL-22 for 3 days. The absorbance values were normalized and results are summarized in Figure 3.9. The results were significantly higher in the treated cells in both SU-DHL-1 and Karpas 299 when compared to the control samples (*P* values, 0.0001, 0.028, respectively, Student's t-test).

To explore the effects on cell growth after IL-22 blockade, all 3 ALK^+ALCL cell lines were serum-starved for 16 hours, and treated daily with 5 µg/mL of IL-22BP protein or 10 µg/mL of IL-22 neutralizing antibody. With the tryphan blue exclusion assay, all ALK^+ALCL cell lines showed a significantly lower viable cell number in the treated samples, whereas no significant differences were detected between the treated and untreated cells in Jurkat and benign lymphocytes (Figure 3.8). SU-DHL-1 and Karpas 299 treated cells showed a decrease in cell viability at approximately Day 4 whereas

SUP-M2 treated cells had a significant decrease in viability at Day 3. The blockade experiments were repeated on SU-DHL-1 and Karpas 299 cells using the MTS assay (Figure 3.9). Similar results were obtained with the use of IL-22BP (*P* values, 0.02 for both SU-DHL-1 and Karpas 299) or IL-22 neutralizing antibody (*P* values, 0.03 and 0.007 for SU-DHL-1 and Karpas 299, respectively) where a decrease in cell growth was observed in the treated cells.

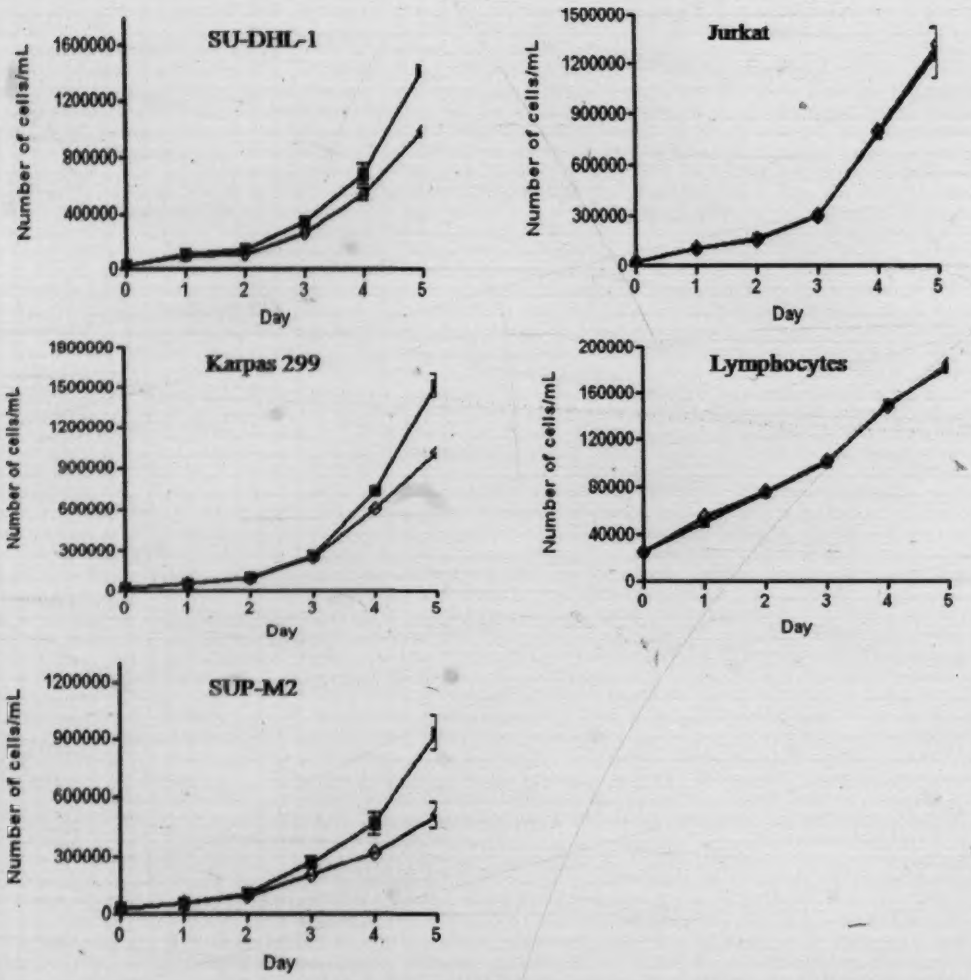

Figure 3.7. IL-22 modulated cell proliferation in ALK$^+$ALCL cell lines. 3 ALK$^+$ALCL cell lines were exposed to 2 conditions: treatment with recombinant IL-22 protein (■) or no treatment (◇). SU-DHL-1, Karpas 299, and SUP-M2 cells were serum starved for 16 hours followed by daily treatment with 10 ng/mL of recombinant IL-22 protein. Daily cell counts were performed using trypan blue exclusion assay. Cell numbers were counted using a hemocytometer at 10X objective. Treatment of 3 ALK$^+$ALCL cell lines with recombinant IL-22 protein induced a marked increase in cell number seen at approximately Day 4. Jurkat cells and benign lymphocytes were included as negative controls and were subjected to the same experimental conditions. No change in cell numbers was observed in either cell types. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of the mean.

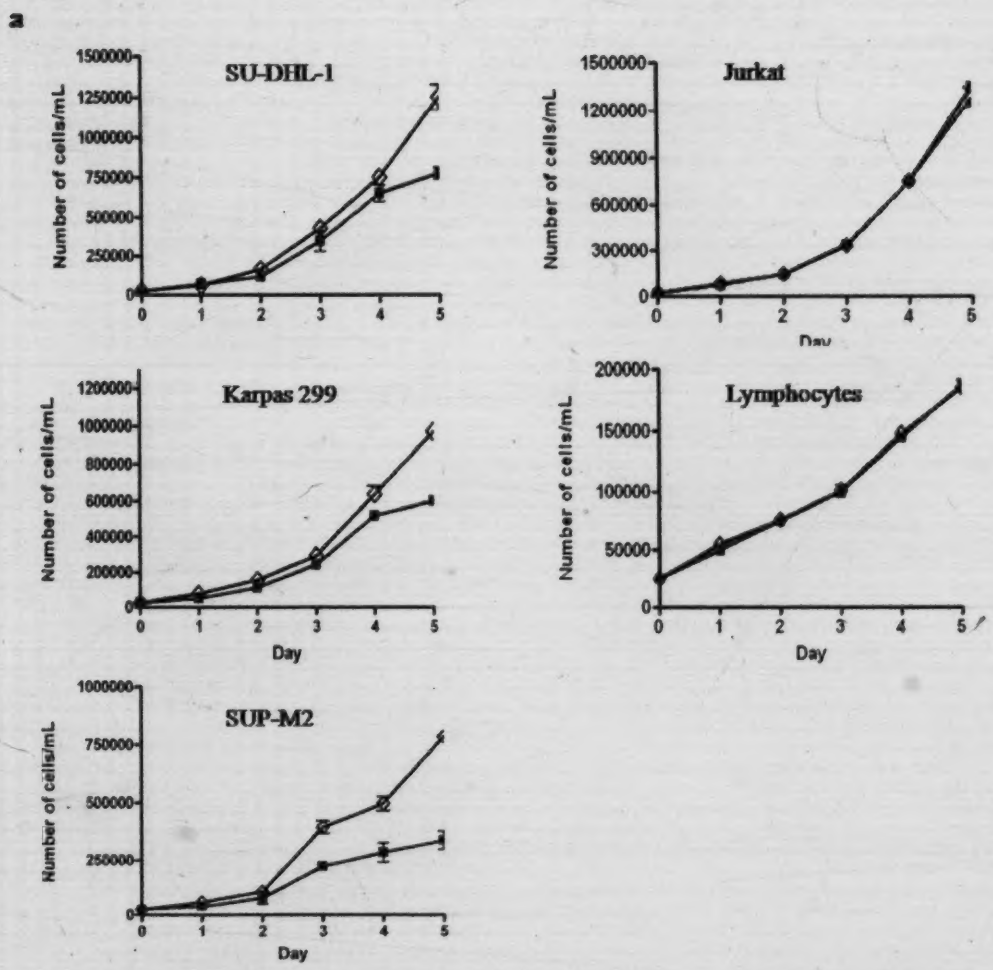

Figure 3.8. IL-22 modulated cell proliferation in ALK$^+$ALCL cell lines. 3 ALK$^+$ALCL cell lines were exposed to 2 conditions: treatment (■) or no treatment (◇). SU-DHL-1, Karpas 299, and SUP-M2 cells were serum starved for 16 hours followed by daily treatment with either recombinant IL-22BP protein (5 μg/mL) or IL-22 neutralizing antibody (10 μg/mL). Daily cell counts were performed using trypan blue exclusion assay. Cell numbers were counted using a hemocytometer at 10X objective. Jurkat cells and benign lymphocytes were included as negative controls and were subjected to the same experimental conditions. No change in cell numbers was observed in either cell types. (a). Treatment of all 3 ALK$^+$ALCL cell lines with recombinant IL-22BP protein resulted in a marked decrease in cell number seen at approximately Day 4 for SU-DHL-1 and Karpas 299. A significant decrease in cell growth was observed at Day 3 for SUP-M2 cells. (Continued next page).

b

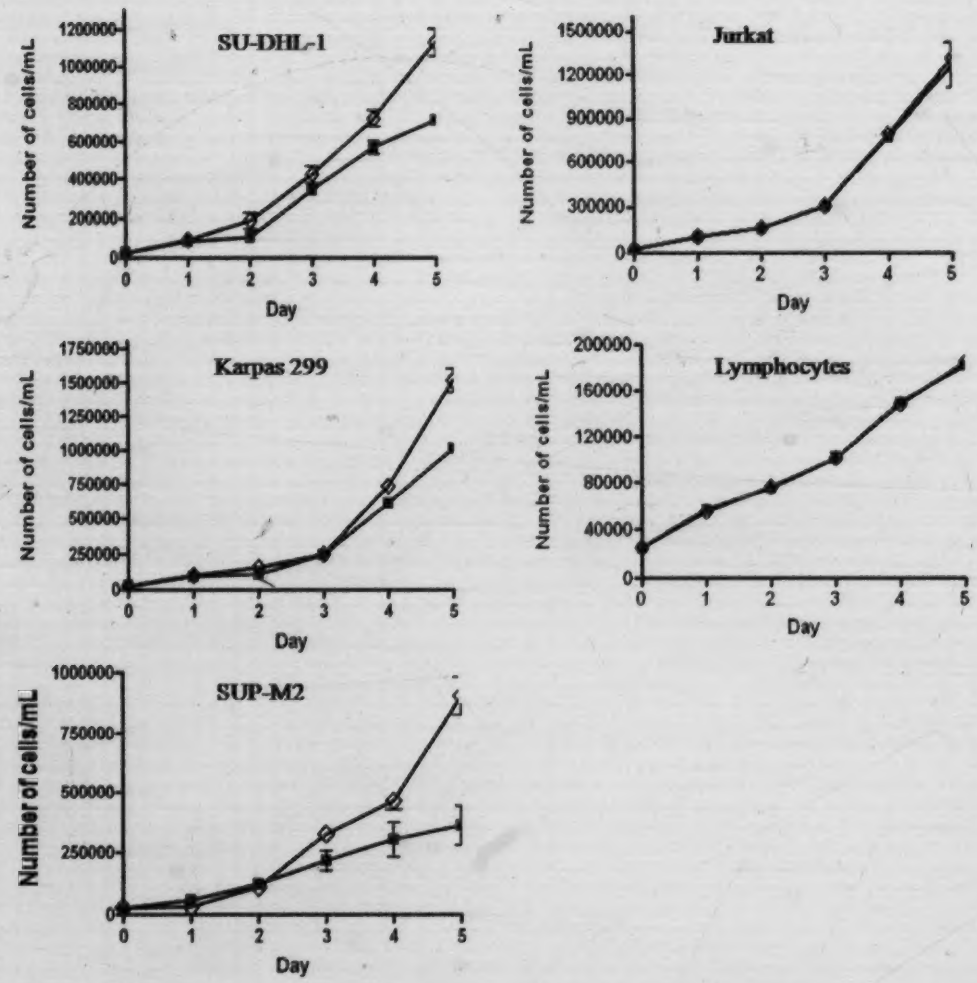

Figure 3.8. (continued) (b). Treatment of all 3 ALK^{+}ALCL cell lines with IL-22 neutralizing antibody resulted in a marked decrease in cell number seen at approximately Day 4 for SU-DHL-1 and Karpas 299. A significant decrease in cell growth was observed at Day 3 for SUP-M2 cells. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of the mean.

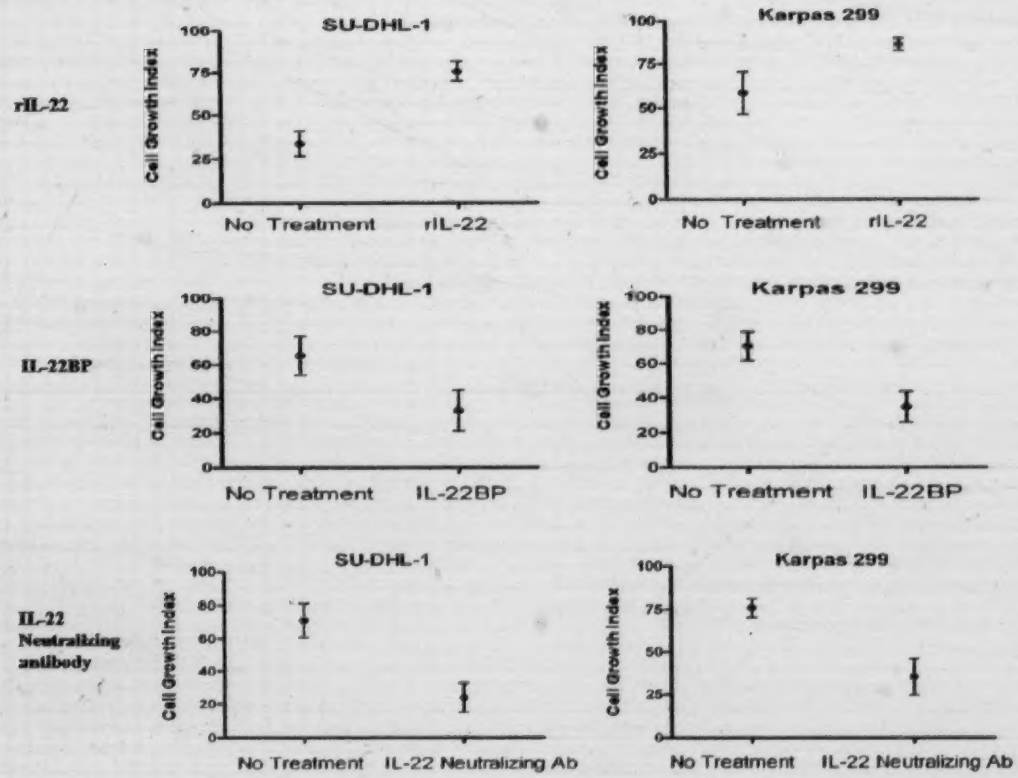

Figure 3.9. IL-22 modulated cell proliferation in ALK$^+$ALCL cell lines using MTS assay. SU-DHL-1 and Karpas 299 cells were exposed to 2 conditions: treatment with recombinant IL-22 protein (10 ng/mL), recombinant IL-22BP protein (5 μg/mL), or IL-22 neutralizing antibody (10 μg/mL) and no treatment. SU-DHL-1 and Karpas 299 cells were plated in a 96-well culture plate and serum starved for 16 hours followed by daily treatment with the three various treatments for 3 days. Cell viability was measured colorimetrically at 450 nm and absorbance values were normalized using the Microplate Manager 5.2.1. Treatment of SU-DHL-1 and Karpas 299 with recombinant IL-22 protein induced a significant increase in the cell growth whereas treatment with IL-22BP and IL-22 neutralizing antibody induced a significant decrease in cell growth. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of the mean.

3.3.3. IL-22 modulates tumorigenicity assessed by colony formation in soft agar

To determine the biological effects and significance of IL-22 autocrine stimulation, we blocked IL-22 using 5 μg/mL of IL-22BP recombinant protein or 10 μg/mL of L-22 neutralizing antibody, and assessed changes in soft agar colony formation. Since only tumorigenic cells have the ability to grow on soft agar, this is an excellent research tool for investigating the tumorigenic potential of cells (Kohlhase et al., 1996). All 3 ALK^+ALCL cell lines, including SU-DHL-1, Karpas 299, and SUP-M2, were serum-starved for 16 hours, treated with either IL-22BP or IL-22 neutralizing antibody for 24 hours, and observed for colony formation for 14 days. Compared to the untreated, ALK^+ALCL cells treated with IL-22BP or the IL-22 neutralizing antibody resulted in a significant decrease in the number of colonies in all three cell lines (*P* value 0.0001 for all 3 cell lines) (Figure 3.10a), with the greatest effect seen in Karpas 299. In contrast, treatment of these cells with recombinant IL-22 proteins (10 ng/mL) for 24 hours led to a significant increase in the number of colonies in SUP-M2 (*P* value 0.0001), SU-DHL-1 (*P* value 0.0001), but not Karpas 299 (*P* value 0.339). The lack of significant effect on Karpas 299 may be related to the fact that these cells produce the highest level of IL-22 among all 3 cell lines (as illustrated in Figure 3.4) and a higher concentration of recombinant IL-22 protein may be required to see a significant difference. Images of cell colony formation with and without IL-22BP recombinant protein treatment are illustrated in Figure 3.10b.

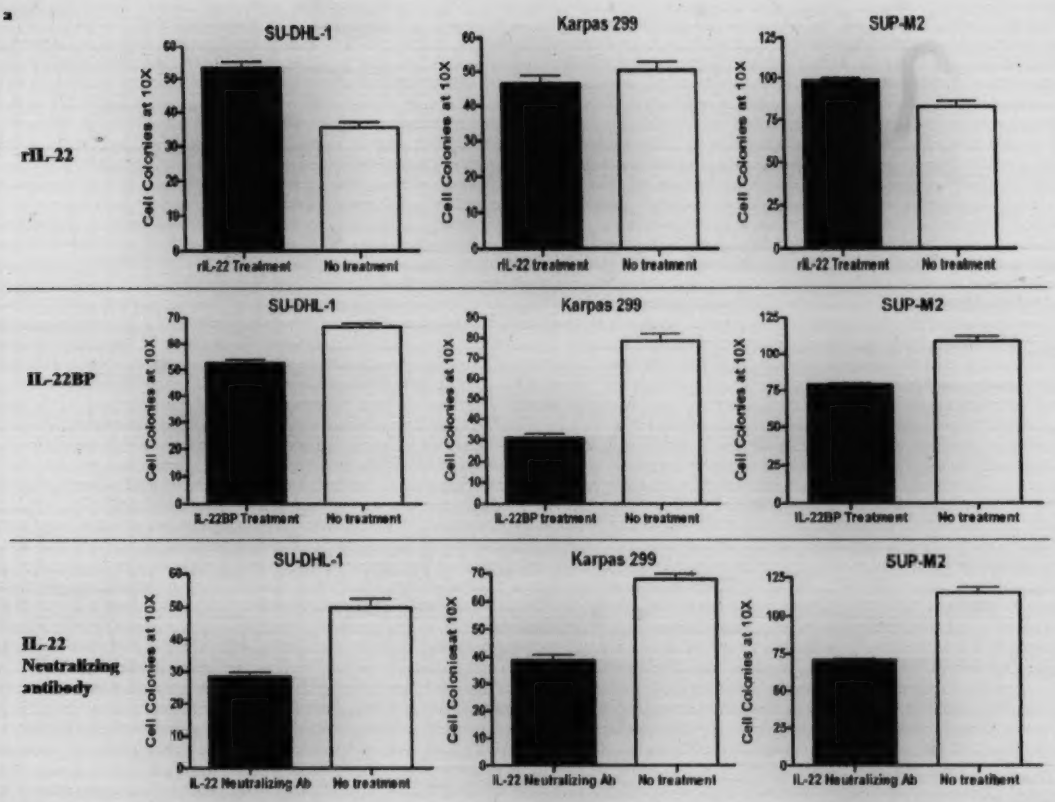

Figure 3.10. IL-22 modulated ALK$^+$ALCL colony formation. SU-DHL-1, Karpas 299 and SUP-M2 cells were exposed to 2 conditions: treatment with recombinant IL-22 protein (10 ng/mL), recombinant IL-22BP protein (5 µg/mL), or IL-22 neutralizing antibody (10 µg/mL) and no treatment. ALK$^+$ALCL cell lines were serum starved for 16 hours followed by treatment with the three different treatments. 20,000 cells were then grown in 0.3% agar/RPMI 1640 for 2 weeks. Cell colonies were then stained with 0.05% crystal violet and quantified using 40X objective. (a). Treatment of ALK$^+$ALCL cell lines with recombinant IL-22 protein induced a significant increase in colony formation in soft agar in SU-DHL-1 and SUP-M2 cells (top panel). There was no significant change in colony number in Karpas 299 cells after two weeks, probably related to the fact that Karpas 299 cell produce a relatively high level of endogenous IL-22. Treatment of the 3 ALK$^+$ALCL cell lines with recombinant IL-22BP protein or IL-22 neutralizing antibody induced a significant decrease in colony formation in soft agar (bottom panels). Karpas 299 cells revealed the greatest difference in colony formation between treated and untreated cells in two weeks (middle panel). Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of the mean. (Continued next page).

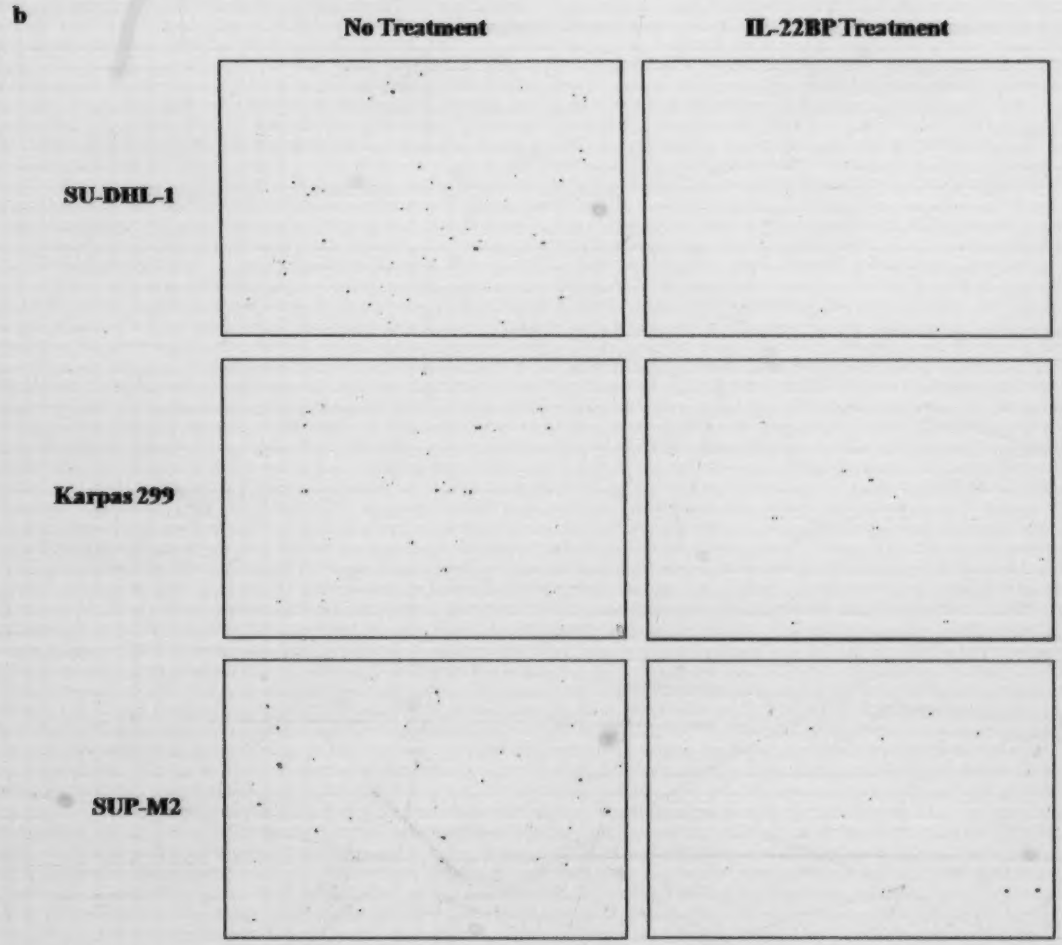

Figure 3.10. (continued). (b). Images of SU-DHL-1, Karpas 299, and SUP-M2 cells before and after treatment with 5 μg/mL of recombinant IL-22BP protein. A significant decrease in cell colonies was observed in the treated samples. Images were photographed using Olympus 1X70 microscope at 20X magnification.

3.3.4. IL-22 modulated STAT3 and MAPK signaling

Because IL-22 have been reported to activate STAT3 in a number of non-hematologic cell lines (Dumoutier et al., 2000; Kotenko et al., 2001; Lejeune et al., 2002; Xie et al., 2000), I sought to determine if IL-22 also contribute to STAT3 activation, a mechanism know to contribute to tumorigenesis in ALK^+ALCL cells. IL-22 blockade using IL-22BP recombinant protein led to a detectable decrease in pSTAT3 in SU-DHL-1 and Karpas 299 (Figure 3.11a illustrates results for Karpas 299). Similar results were observed when these cell lines were treated with an IL-22 neutralizing antibody (Figure 3.11b). Next, cells were treated with IL-22 recombinant protein to determine whether STAT3 would be further stimulated. SU-DHL-1 and Karpas 299 cells were serum-starved for 16 hours prior to treatment with 10 ng/mL of IL-22 protein for 0 and 30 minutes. Figure 3.12a illustrates a detectable increase in the pSTAT3 levels with increasing IL-22 treatment in Karpas 299 cells. HepG2 cells stimulated with recombinant IL-22 protein were included as a positive control with a detectable increase in pSTAT3 observed in the treated cells (Figure 3.12b).

IL-22 is also known to activate major MAPK pathways, including ERK1/2, JNK/SAPK and p38. Interestingly, ERK1/2 kinase has been implicated in ALK^+ALCL through NPM-ALK (Marzec et al., 2007). Therefore, it is of interest to determine whether these kinases can be phosphorylated by the addition of recombinant IL-22 protein ALK^+ALCL cells. As seen in Figure 3.12c, an increase in the phosphorylated form of ERK1/2, JNK/SAPK, and p38 were detected after 30 minute exposure to recombinant Il-22 protein

in Karpas 299. In contrast, the total levels of these three kinases were similar before and after the treatment.

To further evaluate the role of IL-22R1 in ALK^{+}ALCL, IL-22R1 expression was downregulated using siRNA. 24 hours after siRNA transfection, there was a substantial decrease in IL-22R1 cells transfected with IL-22R1 siRNA but not scrambled siRNA (Figure 3.13). Correlating with this change, pSTAT3 was decreased in cells treated with IL-22R1 siRNA.

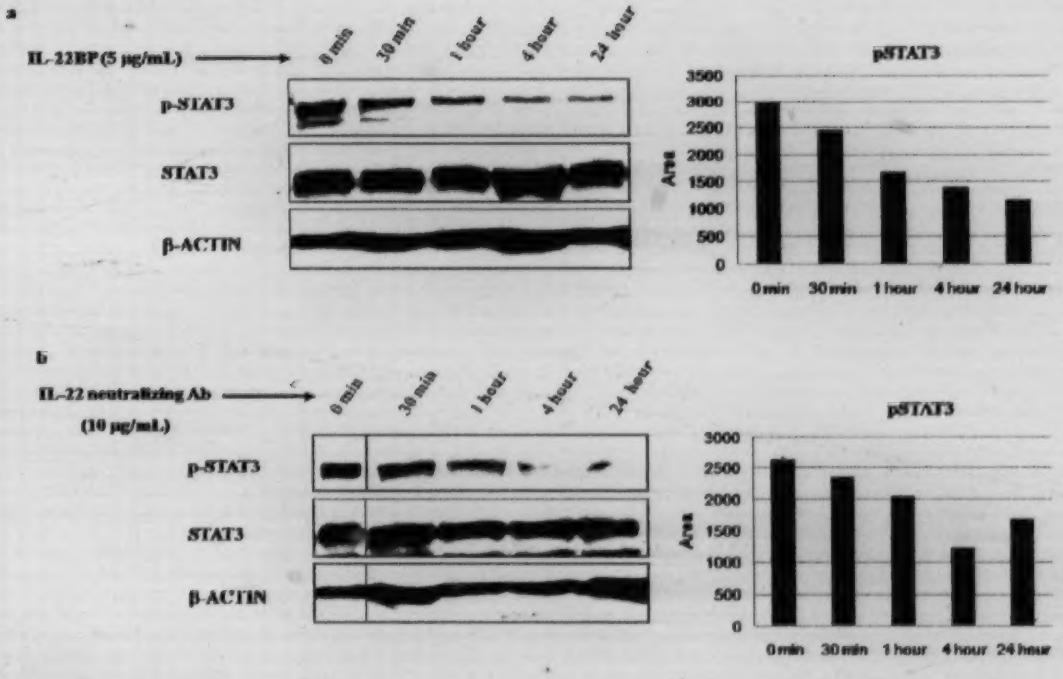

Figure 3.11. Effects of IL-22 on STAT3 signaling pathway. SU-DHL-1, Karpas 299 and SUP-M2 cells were exposed to 2 conditions: treatment with recombinant IL-22BP protein (5 µg/mL) or IL-22 neutralizing antibody (10 µg/mL) and no treatment. ALK^+ALCL cell lines were serum starved for 16 hours followed by treatment with either recombinant IL-22BP protein (5 µg/mL) or IL-22 neutralizing antibody (10 µg/mL) for 0, 30 minutes, 1 hour, 4 hour and 24 hour. Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. Phosphorylated STAT3 was detected using an anti-pSTAT3 antibody followed by detection of the total cellular STAT3 using an anti-STAT3 antibody. Densitometry was performed to quantify protein expression levels of pSTAT3 (figure illustrates results for Karpas 299). (a). Treatment of Karpas 299 with 5 µg/mL recombinant IL-22BP protein markedly decreased pSTAT3 protein level with total STAT3 levels staying the same in all samples. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control. (b). Treatment of Karpas 299 with 10 µg/mL IL-22 neutralizing antibody markedly decreased pSTAT3 protein level with total STAT3 levels staying the same in all samples. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

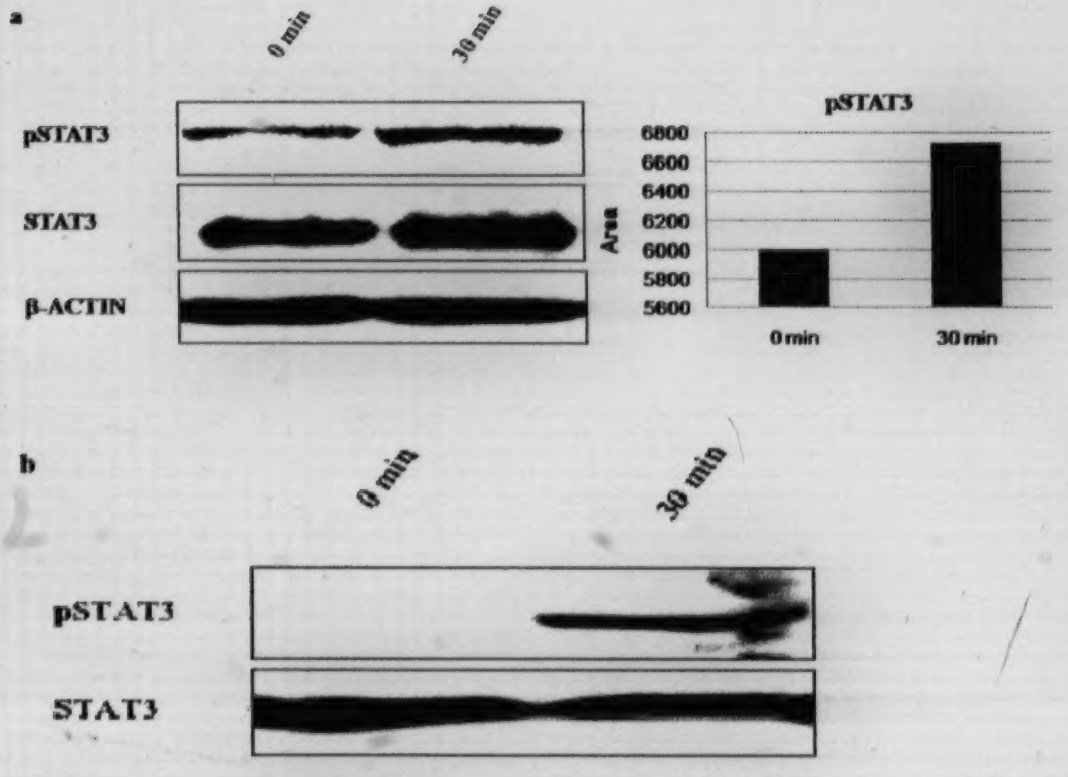

Figure 3.12. Effects of IL-22 on STAT3 and MAPK signaling pathway. SU-DHL-1, Karpas 299 and SUP-M2 cells were exposed to 2 conditions: treatment with recombinant IL-22 protein (10 ng/mL) and no treatment. ALK^+ALCL cell lines were serum starved for 16 hours followed by treatment with 10 ng/mL of recombinant IL-22 protein for 0 and 30 minutes. Densitometry was performed to quantify protein expression levels of pSTAT3 (figure illustrates results for Karpas 299). Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. (a). Phosphorylated STAT3 was detected using an anti-pSTAT3 antibody followed by detection of the total cellular STAT3 using an anti-STAT3 antibody. Treatment of Karpas 299 with recombinant IL-22 protein for 30 minutes markedly increased pSTAT3 protein level with total STAT3 levels staying the same at 0 and 30 minutes. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control. (b). HepG2 was included as a positive control and was subjected to the same experimental treatment with recombinant IL-22 protein for 0 and 30 minutes. pSTAT3 levels were markedly increased in the 30 minute treatment whereas total STAT3 levels stayed the same at 0 and 30 minutes. (Continued next page).

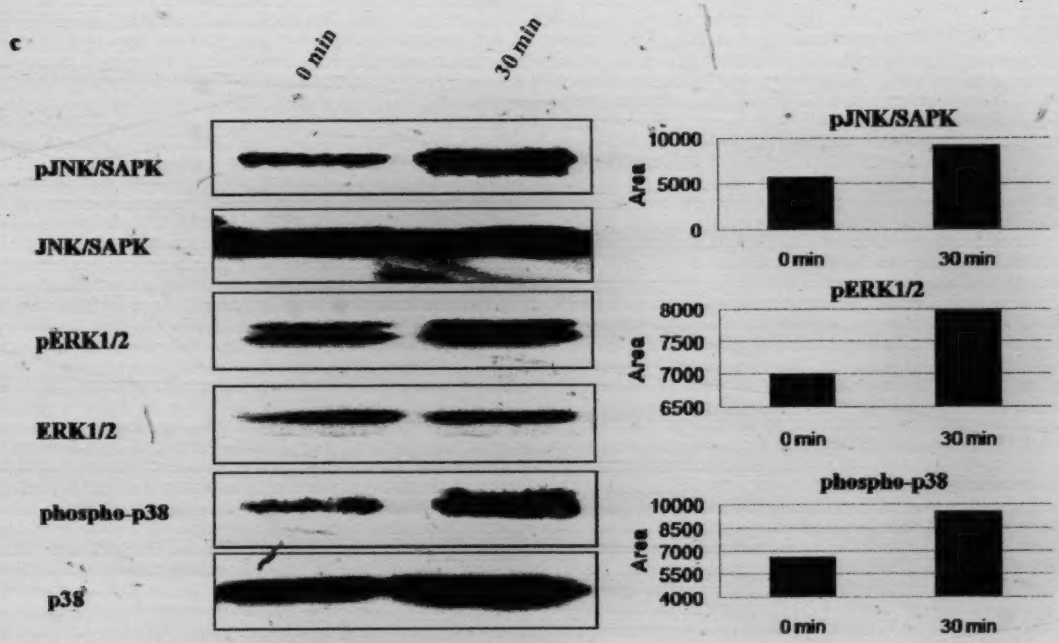

Figure 3.12 (continued) (c). Phosphorylated JNK/SAPK, ERK1/2, and p38 were detected using anti-pJNK/SAPK, anti-pERK1/2, and anti-phospho-p38 followed by detection of the total cellular JNK/SAPK, ERK1/2, and p38. Treatment of Karpas 299 with 10 ng/mL of recombinant IL-22 protein for 30 minutes resulted in an increase in the phosphorylated forms of JNK/SAPK, ERK1/2, and p38 with the total levels of JNK/SAPK, ERK1/2, and p38 not changing between 0 and 30 minute treatments. pSTAT3 protein level with total STAT3 levels staying the same at 0 and 30 minutes. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

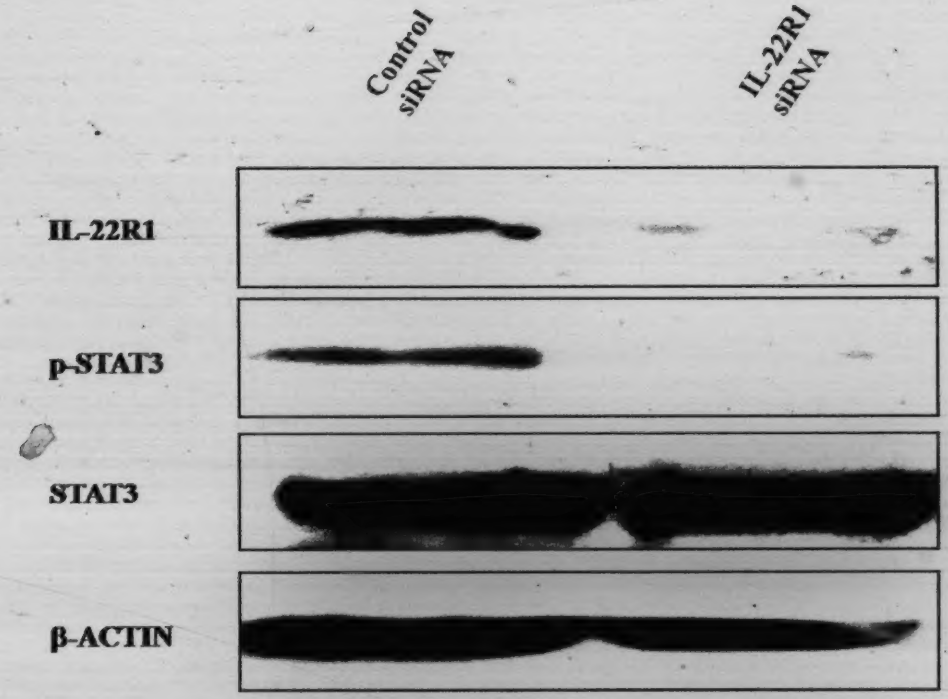

Figure 3.13. IL-22R1 directly up-regulated STAT3 activation. SU-DHL-1 cells were transfected with either IL-22R1 siRNA or control siRNA. After 24 hour incubation, lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. IL-22R1 was detected using an anti-IL-22R1 antibody. Phosphorylated STAT3 was detected using an anti-pSTAT3 antibody followed by detection of the total cellular STAT3 using an anti-STAT3 antibody. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control. Downregulation of IL-22R1 using siRNA was sufficient in reducing pSTAT3 expression by Western blot analysis. In contrast, the control siRNA had no change in IL-22R1 and pSTAT3 expression.

3.3.5. NPM-ALK induced IL-22R1 expression

Because NPM-ALK is the characteristic oncoprotein in this tumor type, I asked if NPM-ALK is responsible for the aberrant expression of IL-22R1 in ALK^+ALCL. Jurkat cells, a T-lymphoblast cell line that lacks IL-22R1 expression, was subjected to transfection with either *NPM-ALK* vector or empty vector. The expression of the NPM-ALK transgene was confirmed by RT-PCR (Figure 3.14a) in cells transfected with *NPM-ALK* but not the negative control vector. In cells transfected with *NPM-ALK*, IL-22R1 protein expression was detectable by Western blots (Figure 3.14b). In contrast, Jurkat cells transfected with the empty vector had no detectable levels of Il-22R1 protein expression.

To assess the ability of IL-22R1 to stimulate STAT3 activation by NPM-ALK, Jurkat cells transfected with either NPM-ALK or an empty vector were treated with IL-22 for 30 minutes and subjected to Western blot. Interestingly, only cells transfected with NPM-ALK but not the empty vector showed detectable pSTAT3 after treatment with IL-22 (Figure 3.15). This finding correlates with Figure 3.14b where IL-22R1 expression was stimulated by NPM-ALK transfection.

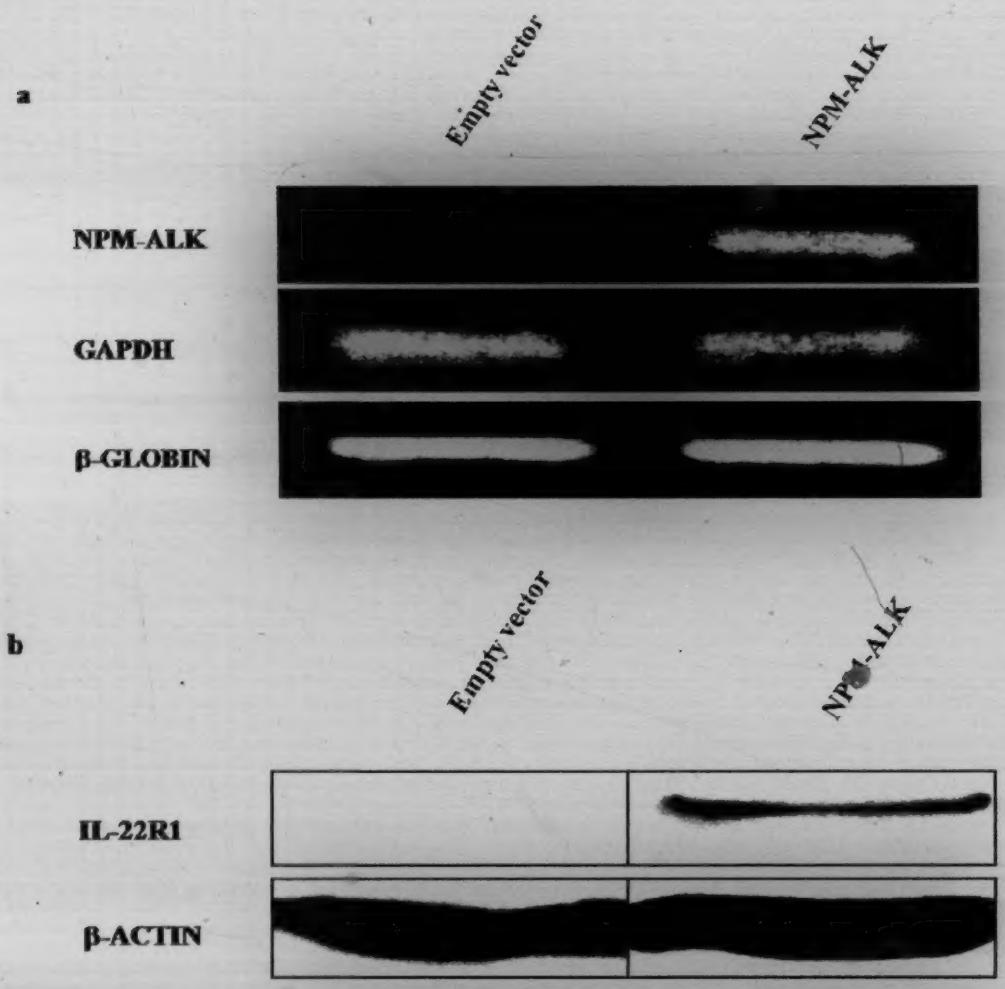

Figure 3.14. Induction of IL-22R1 expression in NPM-ALK activated cell line. Jurkat cells were transfected with 1 μg of NPM-ALK vector or an empty vector. (a). RT-PCR studies confirmed the expression of *NPM-ALK* in Jurkat cells after transfection. In contrast transfection with the empty vector had no detectable levels of NPM-ALK. GAPDH and β-globin were included as internal controls. (b). Western blot analysis was performed to detect IL-22R1 in the NPM-ALK-transfected cells. Protein lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. IL-22R1 was detected using an anti-IL-22R1 antibody. Transfection of Jurkat cells with *NPM-ALK* induced expression of IL-22R1. In contrast, no IL-22R1 expression was detected in Jurkat cells transfected with an empty vector. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

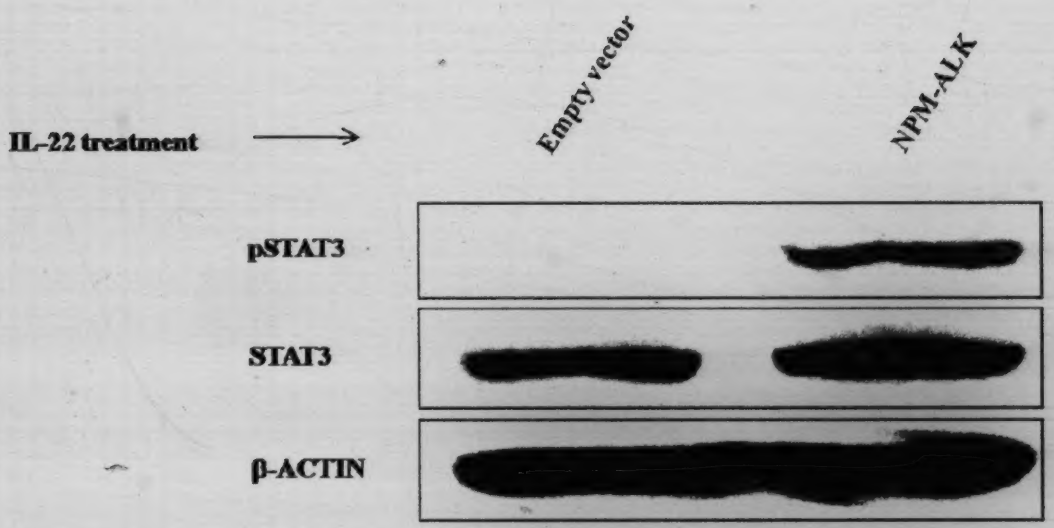

Figure 3.15. Induction of STAT3 activation in NPM-ALK activated cell line. Jurkat cells were transfected with 1 μg of NPM-ALK vector or an empty vector and treated with 10 ng/mL of recombinant IL-22 protein for 30 minutes. Protein lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. Phosphorylated STAT3 was detected using an anti-pSTAT3 antibody followed by detection of the total cellular STAT3 using an anti-STAT3 antibody. pSTAT3 was detected in Jurkat cells transfected with NPM-ALK. In contrast, no pSTAT3 expression was detected in Jurkat cells transfected with an empty vector. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

3.3. Discussion

IL-22, a recently discovered cytokine (Dumoutier et al., 2000), is a member of the IL-10 family proteins largely expressed by activated T- and NK-cells (Kotenko, 2002); (Brand et al., 2007). The expression of one of the IL-22R heterodimeric proteins, namely IL-22R1, is relatively restricted and found largely on epithelial cells from various mucosal surfaces. Characteristically, IL-22R1 is not expressed in benign lymphoid cells, resting and activated monocytes, and dendritic cells (Wolk et al., 2002; Wolk et al., 2005; Wolk et al., 2006). *In vitro*, IL-22 promotes cell proliferation and anti-apoptotic effects in various epithelial cell lines. It also has been shown to activate a number of signaling pathways in epithelial cells, including those of STAT3, MAPK and AKT (Lejeune et al., 2002; Weber et al., 2006). Similar to STAT3 and ERK1/2, AKT signaling pathway is also involved in cell proliferation and apoptosis (Dauer et al., 2005). Based on these findings, it is widely believed that IL-22 plays an important role in innate immunity (Gurney, 2004). In this chapter, I sought to identify a novel autocrine stimulatory pathway that may possibly contribute to the pathogenesis of ALK^+ALCL through activation of the STAT3 signaling pathway.

One of the significant findings of this study is that of aberrant expression of IL-22R1 in ALK^+ALCL, a type of mature T-cell neoplasm. The expression of IL-22R1, initially identified in oligonucleotide arrays, was confirmed by several different methods such as RT-PCR, Western blots, confocal microscopy and immunohistochemistry. The expression of IL-22R1 in ALK^+ALCL is a consistent finding, since it is readily detectable in all ALK^+ALCL tumors and cell lines examined. I also identified consistent expression

of IL-22 by these tumors, a finding that is not too surprising considering that ALK^+ALCL is a T-cell neoplasm. A recently published study also reported the presence of IL-22 in ALK^+ALCL (Lamant et al., 2006). In view of the existence of a naturally occurring IL-22R decoy, namely IL-22BP, I also performed RT-PCR to detect the expression of this protein in ALK^+ALCL cell lines and the results indicate that this regulatory protein is consistently absent in these cells. Taken together, the findings in this chapter strongly suggest that the IL-22 autocrine stimulatory pathway is functional and un-checked in ALK^+ALCL. Functional assays including the soft agar colony formation assay support the concept that the IL-22 autocrine pathway is biological significant in ALK^+ALCL and contributes to the tumorigenicity.

In keeping with the previous findings of IL-22 mediated activation of the STAT3 pathway in a number of epithelial cell types (Lejeune et al., 2002; Nagalakshmi et al., 2004; Weber et al., 2006), I found that STAT3 activation can be enhanced by IL-22 in ALK^+ALCL cells. This finding is of great interest, since constitutive activation of STAT3 has been shown to be crucial to the pathogenesis of ALK^+ALCL. Specifically, blockade of STAT3 signaling induces apoptosis and cell cycle arrest in ALK^+ALCL cell lines (Amin, 2004). In another study, Chiarle *et al.* showed that oncogenic effects of NPM-ALK is dependent on STAT3 activation (Chiarle et al., 2005). Thus, enhancement of STAT3 activation in this cell-type may likely explain the increased tumorigenicity induced by IL-22. Our finding also further support the concept that STAT3 activation in ALK^+ALCL is multi-factorial, as first pointed out by Zhang *et al.* (Zhang et al., 2002). In addition to NPM-ALK, it has been reported that Src (Cussac et al., 2004), JAK3

(Amin and Lai, 2007; Amin et al., 2003b; Han et al., 2006; Zhang et al., 2002), and IL-9 (Xin 2006) contributes to deregulate this signaling pathway. The relatively high levels of IL-9 production in this cell-type may explain the production of IL-22, as IL-22 is well known to be an IL-9-inducible cytokine (Dumoutier et al., 2000). Our lab has also recently showed that the sustained STAT3 activation is potentiated by the loss of SHP1, a tyrosine phosphatase that normally provides negative feedback to the JAK3/STAT3 signaling exist in ALK^+ALCL (Han et al., 2006).

Of note, other than the STAT3 signaling pathway, IL-22 has been reported to activate the three major MAPK pathways that converge on extracellular signal-regulated kinases, including ERK1/2, JNK, and p38 kinase pathways (Andoh et al., 2005; Ikeuchi et al., 2005; Lejeune et al., 2002; Nagai et al., 2007; Radaeva et al., 2004). All of these signaling pathways play pivotal role(s) in a variety of cellular responses, including cell proliferation, cell differentiation and cell survival (Nagai et al., 2007; Torii et al., 2006). Specifically, ERK activation is known to downregulate anti-proliferative genes to allow successful G_1 phase progression (Yamamoto et al., 2006). The JNK and p38 kinase signaling pathways are involved in many environmental stress responses, and has been reported to regulate apoptosis (Davis, 2000; Tournier et al., 2000; Wang et al., 2004). These MAPK pathways, which have been implicated in transformation and tumor progression in many human cancers (Du et al., 2004; Nagata and Todokoro, 1999; Ono and Han, 2000), were confirmed to be activated by IL-22 in ALK^+ALCL cell lines and stimulation of these pathways likely further promotes the tumorigenic effects of IL-22.

Thus, the finding that IL-22 induces up-regulation of the phosphorylated/active form of ERK, JNK, and p38 is significant.

Since NPM-ALK plays a key role in the pathogenesis of ALK^+ALCL, I investigated if NPM-ALK is directly responsible for the aberrant expression of IL-22R1 expression. Transfection experiments using Jurkat cells, a T-lymphoblast cell line that lack IL-22R1 expression, strongly support that NPM-ALK expression can induce IL-22R1 expression, which in turn can activate STAT3 (Figure 3.13, 3.14). Thus, these results have clearly shown that NPM-ALK expression can convert an 'IL-22 unresponsive phenotype' into an 'IL-22 responsive phenotype'. The mechanism by which NPM-ALK mediates this effect is unclear, but it is likely that specific signaling pathways may be implicated. As previously mentioned, NPM-ALK has been reported to induce the phosphorylation of ERK1/2 in ALK^+ ALCL cell lines (Marzec et al., 2007). Interestingly, scanning of the promoter region of *IL-22R1* revealed elk-1 and RREB-1 transcription factors. Elk-1 is known to be phosphorylated by ERK1/2 (Conner et al., 2003; Zhang et al., 2006), and RREB-1 is involved in Ras signal transduction (Thiagalingam et al., 1996), which is known to activate the MAPK/ERK kinase (Mor and Philips, 2006). This may provide another possible explanation as to the aberrant expression of IL-22R1 in ALK^+ALCL, where there is a positive feedback loop between ERK1/2 phosphorylation and IL-22R1 activation. Figure 3.16 illustrates a hypothetical model of IL-22 autocrine pathway in ALK^+ALCL.

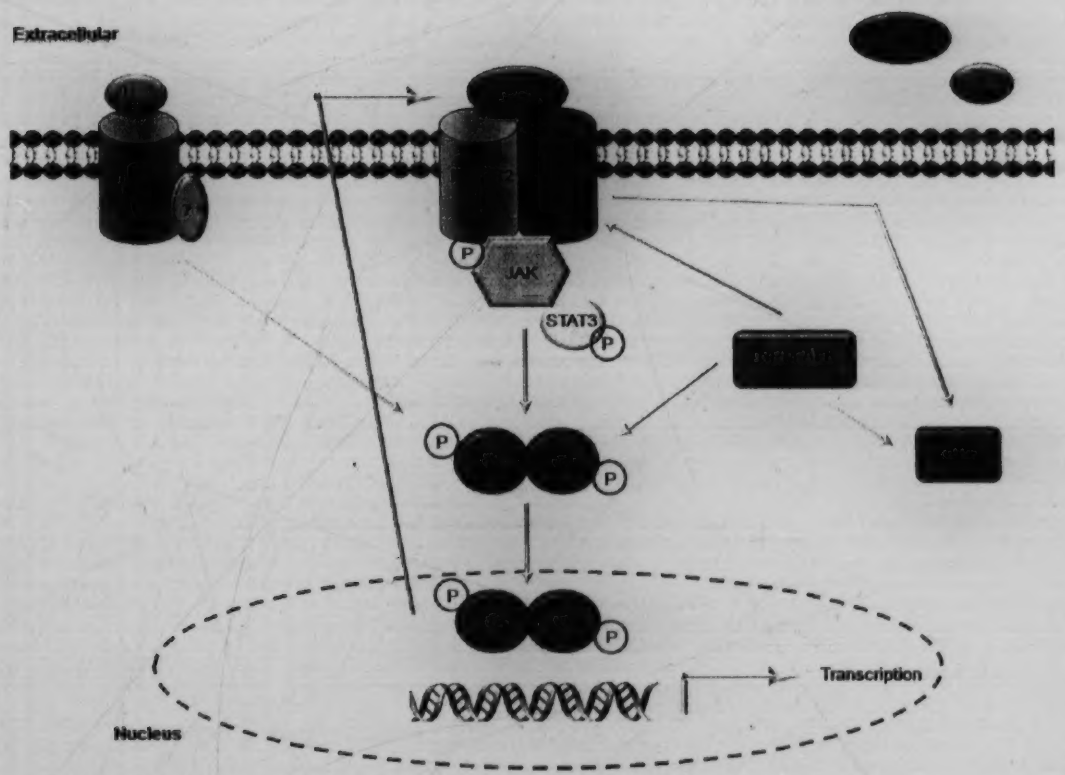

Figure 3.16. A hypothetical model of the IL-22 autocrine pathway in ALK$^+$ALCL. IL-22 is an inflammatory cytokine that is produced by T-cells. The cytokine may bind to IL-22BP which will prevent its binding to IL-22R1. For IL-22 to exert its biological function, it must bind to IL-22R1 and IL-10R2 simultaneously. In ALK$^+$ALCL, the IL-22 receptor likely activates JAK3, which contributes to tyrosine phosphorylation and activation of STAT3. Once activated, the STAT3 homodimers migrate to the nucleus to modulate transcription of specific genes, promoting cell survival and growth. IL-22 is also known to activate the MAPK pathway, which has been recently shown to be important in the pathogenesis of ALK$^+$ALCL. NPM-ALK, a tyrosine kinase involved in the activation of STAT3 and MAPK pathway in ALK$^+$ALCL, is implicated in the induction of IL-22R1 expression. IL-9, a cytokine known to induce IL-22 secretion, has been recently shown to be produced by ALK$^+$ALCL.

Multiple experiments in this study have identified an IL-22 autocrine stimulatory pathway that promotes tumorigenicity and cell growth in ALK^+ALCL. In contrast, another study reported that IL-22 mediates reduction in tumor growth and cell cycle arrest via downregulating ERK1/2 and AKT activation in murine breast cancer cell lines (Weber et al., 2006). I believe that there are at least three possible explanations for this discrepancy. First, human ALK^+ALCL cell lines were used throughout this chapter whereas the paper by Weber *et al.* primarily involved murine breast cancer cell lines. Therefore, the effects of IL-22 may be specific to cell-type, and possibly, species. Second, it has been reported that the biological effects of IL-22 are concentration-dependent. In one study, a relatively low IL-22 concentration led to cell proliferation, whereas a relatively high IL-22 concentration suppressed cell proliferation in HepG2 cells (Radaeva et al., 2004). In this regard, Weber *et al.* employed 50 ng/mL of IL-22, a concentration that is 5-fold higher than what is used in most studies as well as this chapter (Weber et al., 2006). Through screening of a cohort of primary breast tumors for IL-22 and its corresponding receptors by RT-PCR, I have identified IL-22R1 and IL-10R2 in 10/10 cases, IL-22 in 0/10 cases and IL-22BP in 7/10 breast tumors. With this finding, the third possible reason for discrepancy between the findings in this chapter and the study by Weber *et al.* is the strong presence of the receptor antagonist IL-22BP in breast cancer, which may have prevented recombinant IL-22 protein from exerting its pro-stimulatory function.

Of note, a recent paper using cDNA microarray has demonstrated that ALK^-ALCL produces IL-22 (Lamant et al., 2006) however, the study did not investigate the

expression of IL-22R1. More recent studies in our laboratory have shown that the aberrant expression of IL-22R1 is not restricted to ALK^+ALCL. In fact, one of the ALK- ALCL cell lines, MAC-2A, showed IL-22R1 expression (data not shown). Thus, mechanisms other than NPM-ALK likely induce aberrant IL-22R1 expression in lymphoma cells. It is highly likely that the IL-22 autocrine stimulatory pathway also carry important biological significance in the lymphomagenesis of ALK^-ALCL, and this area warrants further studies.

In conclusion, I present the first evidence of aberrant expression of IL-22R1 in a human lymphoid malignancy. This aberrancy, which is linked to the oncoprotein NPM-ALK, creates an abnormal autocrine stimulatory pathway that promotes tumorigenesis, likely by activating multiple signaling pathways including that of STAT3 and MAPK. These findings suggest that IL-22 signaling pathway may be a useful therapeutic target in ALK^+ALCL.

3.5. References

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Chapter 4 ♦

IL-21 contributes to JAK3/STAT3 activation and promotes cell growth in ALK-positive anaplastic large cell lymphoma

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4.1. Introduction

Anaplastic lymphoma kinase-expressing anaplastic large-cell lymphoma (ALK^+ALCL) is a subtype of T/null-cell non-Hodgkin's lymphoma characterized by a constellation of pathologic and clinical features (Amin and Lai, 2007). The aberrant expression of ALK in most of these tumors is the result of the reciprocal chromosomal translocation, t(2;5)(p23;q35), which leads to the fusion of the *nucleophosmin* (*NPM*) gene at 5q35 with the *anaplastic lymphoma kinase* (*ALK*) gene at 2p23 (Morris et al., 1994; Shiota et al., 1994b). It is widely accepted that NPM-ALK directly contributes to lymphomagenesis (Amin and Lai, 2007). Accumulating data suggest that NPM-ALK mediates lymphomagenesis by virtue of its constitutively active tyrosine kinase activity that is embedded in the ALK portion of this fusion protein. Through their interactions with NPM-ALK, multiple signaling proteins became tyrosine phosphorylated and thus, constitutively activated (Amin et al., 2003b; Bischof et al., 1997; Chiarle et al., 2005; Fujimoto et al., 1996; Slupianek et al., 2001). JAK3/STAT3 is a well characterized signaling pathway in ALK^+ALCL. JAK3 is pathogenetically important in ALK^+ALCL, since inhibition of JAK3 reduces the ALK tyrosine kinase activity, downregulates STAT3 activation, and induces apoptosis and G_1 cell-cycle arrest in ALK^+ALCL cell lines (Amin et al., 2003b). One of the JAK3 downstream mediators is STAT3, a family member of latent transcription factors activated in response to cytokines and growth factors (Bowman et al., 2000; Darnell, 1997). Both JAK3 and STAT3 are constitutively activated in ALK^+ALCL [9,21,22]. STAT3 is an oncogene (Bromberg et al., 1999), and a variety of human cancer types express constitutively active STAT3 (Burke et al., 2001;

Levy and Darnell, 2002; Lin et al., 2000; Ma XT, 2004). STAT3 is known to promote oncogenesis by modulating the expression of many important regulatory proteins involved in apoptosis and cell cycle, such as c-Jun, c-Myc, Bcl-xL, Bcl-2, Mcl-1, survivin, cyclins, p21, and p27 (Bowman et al., 2000; Coqueret and Gascan, 2000; Epling-Burnette et al., 2001; Grad et al., 2000; Mahboubi et al., 2001). Accumulating evidence supports the concept that NPM-ALK mediates its oncogenic effects via STAT3 activation (Chiarle et al., 2005; Khoury JD, 2003; Zamo et al., 2002; Zhang et al., 2002), and blockade of STAT3 in ALK^+ALCL cell lines results in significant apoptosis and cell cycle arrest (Amin et al., 2004). While NPM-ALK plays a direct role in activating STAT3, sustained activation of this protein appears to be multi-factorial in ALK^+ALCL, and the mechanism has been attributed to Src and the loss of various negative feedback systems such as SHP1, a tyrosine phosphatase (Amin et al., 2003b; Fujitani et al., 1997; Han et al., 2006; Qiu et al., 2006). JAK3, the physiologic activator of STAT3 which also contributes to STAT3 activation in ALK^+ALCL, was recently found to be attributed to autocrine cytokine stimulation, namely IL-9 [25].

Interleukin-21 (IL-21), a newly discovered cytokine (Parrish-Novak et al., 2000), is expressed exclusively by CD4-positive T cells and known to regulate the functions of T-cells, B-cells, natural killer cells and myeloid cells (Asao et al., 2001; Habib et al., 2002). IL-21 is considered a class I cytokine and it has a significant homology to IL-2, IL-4, and IL-15 (Parrish-Novak et al., 2000). All of the class I cytokines, including IL-9, IL-15, and IL-21, have receptors that contains the IL-2 common γ-chain (γ_c). Their biological importance is highlighted by the phenotype identified in the JAK3-deficient severe

combined immunodeficient (SCID) mice as well as X-linked SCID mice in which the gene encoding γ_c is mutated (Leonard, 2001; Macchi et al., 1995; Russell et al., 1995). IL-21-mediated cell signaling requires heterodimerization of its receptors, γ_c and IL-21R, which is normally expressed on B-cells, T-cells and natural killer cells (Habib et al., 2002). IL-21 induces activation of both JAK1 and JAK3, which then initiate STAT1 and STAT3 signal transduction and stimulate various cellular responses in a cell-type specific manner (Asao et al., 2001; Habib et al., 2003; Strengell et al., 2002; Suto et al., 2002). For example, IL-21 has a pro-apoptotic effect on B-cells (Mehta et al., 2003) but a proliferative effect on T cells (Li et al., 2005; Zeng et al., 2005). While IL-21 has been reported to promote cell growth and proliferation in some cancer types including myeloma and adult T-cell leukemia (Akamatsu et al., 2007; Brenne et al., 2002; Ueda et al., 2005), it has been shown to induce apoptosis in follicular lymphoma (Akamatsu et al., 2007).

In view of the biological relevance of JAK3/STAT3 in ALK^+ALCL and the link between IL-21 and the JAK3/STAT3 signaling pathway, I hypothesize that IL-21 may contribute to JAK3/STAT3 activation and promote tumorigenesis in ALK^+ALCL, in a similar manner as IL-9 as described previously by our group (Qiu et al., 2006). The biological relevance and importance of IL-21 have not been previously examined in ALK^+ALCL. In our initial study using oligonucleotide microarrays to study the cytokine expression in ALK^+ALCL, aside from the IL-22 complex that was described in chapter 3, both IL-21R and IL-21 were found to be frequently expressed in this cell type. In this chapter, the expression of IL-21 and IL-21R in ALK^+ALCL cell lines and tumors was

comprehensively evaluated. The biological effects of IL-21 in ALK^+ALCL cells were also assessed, with a focus on the JAK3/STAT3 signaling.

4.2. Materials and Methods

4.2.1. Cell lines and Culture

Three ALK^+ALCL cell lines, SU-DHL-1, Karpas 299, and SUP-M2 were used in this study. All three cell lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). HepG2 (ATCC, HB-8065) and MDA-MB-231 (ATCC HTB-26) were also used in this study and was maintained in DMEM (Sigma-Aldrich). Both culture media were enriched with 10% FBS (Gibco) and antibiotics (10,000 units/mL penicillin G, 10,000 µg/mL streptomycin, Gibco). All cells were grown at 37°C in 5% CO_2.

4.2.2. Treatment of Cells

For cytokine experiment, cells (1 X 10^6 cells/mL) were washed twice with PBS and plated in 6-well culture plates (Bectin Dickinson Labware) with serum-free media for 16 hours. Cells were treated with 10 ng/mL of Recombinant IL-21 protein (Sigma-Aldrich), for 0 and 30 min and harvested for western blot.

4.2.3. Western Blot Analysis and Antibodies

Western blot analysis was performed using standard techniques. Briefly, cells were collected, centrifuged at 300*g* for 10 minutes, and washed twice with PBS. Lysis buffer

(20 mM tris-HCl, pH 7.5, 5.0 mM EDTA, 40.0 μg/mL leupeptin, 1 μM pepstatin, 1 mM AEBSF) was added to the cell pellet for 30 minutes on ice followed by centrifugation at 14,000*g* for 10 minute at 4°C. The supernatant was removed and 50-100 μg of protein was run on SDS-PAGE. Protein quantification was carried out using the Bradford assay according to the manufacturer's protocol (Biorad). After the proteins were transferred to nitrocellulose membranes, the membranes were blocked with 5% milk in TBS buffer (Sigma) and 0.05% Tween-20 (Calbiochem), and then incubated with primary antibodies overnight followed by 1 hour incubation with horseradish peroxidase-conjugated secondary antibody (1:10000) (Jackson Immunoresearch Laboratories). The membranes were washed in TBS with 0.05% Tween-20 for 30 minutes between steps. Proteins were detected using the enhanced chemiluminescence detection kit (Amersham Life Sciences). The number in parenthesis after each antibody listed indicates the dilution at which it was used. Antibodies used included: Antibodies used included: rabbit polyclonal anti-human STAT3 (1:500, Santa Cruz), mouse monoclonal anti-human pSTAT3 (1:500, Santa Cruz), goat polyclonal anti-human pJAK3 (1:500, Santa Cruz), rabbit polyclonal anti-human JAK3 (1:500, Santa Cruz), rabbit monoclonal anti-human pERK1/2 (1:1000, Cell Signaling), mouse monoclonal anti-human ERK1/2 (1:1000, Cell Signaling), rabbit polyclonal anti-human IL-21R (1:1000, Sigma), and mouse monoclonal anti-human β-actin (1:3000, Sigma-Aldrich).

4.2.4. Immunofluorescence Staining and Confocal Microscopy

Immunofluorescence was performed using standard techniques. Briefly, 1 X 10^6 cells cells grown on coverslip in a 6-well plate were fixed with 4% paraformaldehyde in PBS.

Cells were rinsed with 1X PBS, permeabilized with PBS-0.5% triton X-100 for 5 minutes, and rinsed twice with 1X PBS. Cells were rinsed three times with 1X PBS, incubated with 30 µL of anti-IL-21R (1:50) (Sigma-aldrich) antibody overnight, followed by rinsing three times with 1X PBS. IgG antibody was included as a negative control. After Incubation with 25 µL of Alexa 488 goat anti-rabbit secondary antibody (1:250) for 1 hour, cells were rinsed with PBS and mounting media (Sigma-aldrich) was added to the slides. Cells were visualized with a Cells were visualized and imaged with a Zeiss LSM 510 confocal microscope (Oberkochen, Germany). Argon laser with a 488 nm wavelength was used to visualize IL-22R1 at 40X objective and images were analyzed using the Zeiss LSM 5 image browser.

4.2.5. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted from SU-DHL-1, Karpas 299, SUP-M2 and HepG2 cell lines using TRIzol extraction according to manufacturer's protocol (Gibco). RT was performed using 500 ng total RNA in a first strand cDNA synthesis reaction with superscript reverse transcriptase as recommended by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA). Primer pairs were designed by Primer3 Input 0.4.0 to detect IL-21 and IL-21R. GAPDH was included as an internal control. PCR was performed by adding 1 µL RT product into 25 µL volume reaction containing 1X buffer, 200 µM of each dNTPs, oligonucleotide primer, and 0.2 U Ampli*Taq* polymerase. The primer sequences and PCR cycles are shown in Table 4.1. For DNA amplification, cDNA was denatured at 94°C for 1 min, and then subjected to primer annealing at 58°C for 1 min, and then subjected to DNA extension at 72°C for 1 min for 35 cycles in a

thermal cycler (Applied Biosystems, Foster City, CA, USA). Amplified product were analyzed by DNA gel electrophoresis in 1% agarose and visualized by the Alpha Imager 3400 (Alpha Innotech).

Table 4.1. Primer Sequence and PCR conditions

	Sense Primers	Antisense Primers	Product size (bp)
IL-21	GGCAACATGGAGAGGATTGT	AAGCAGGAAAAAGCTGACCA	240
IL-21R	TCTACATGCTGAAGGGCAAG	TCACTCCATTCACTCCAGGT	315
GAPDH	AAGGTCATCCCTGAGCTGAA	CCCTGTTGCTGTAGCCAAAT	316

4.2.6. Cell growth assays

To determine cell viability in cells treated with recombinant IL-21 protein, daily cells counts was performed. For cell count, 25,000 cells were plated in 24-well culture plates with medium containing 5% FBS. Recombinant IL-21 was added daily and cells were counted daily using trypan blue exclusion assay (Gibco).

4.2.7. siRNA Transfection

Karpas 299 cells (1 X 10^6 cells in 1 mL of culture medium) were transfected with 100 pmol of IL-21R or scrambled siRNA (Qiagen) using the Nucleofector Kit (Amaxa) according to the manufacturer's protocol. The IL-21R target sequence is: 5'-CCCGGTCATCTTTCAGACCCA-3'. Cells were harvested 24 hours after the transfection.

4.2.8. MTS Assay

To determine the effect of SALL4 on cell viability, Karpas 299 cells were transfected with either IL-21R siRNA or control siRNA and the MTS assay (Promega) was performed after 72 hours. 5,000 cells were seeded in 96-well culture plates and cell viability was measured daily using a colorimetric Microplate reader (Biorad) at 450nm and absorbance values were normalized using the Microplate Manager 5.2.1 (Biorad).

4.2.9. ALK^+ALCL tumors and immunohistochemistry

10 paraffin-embedded lymph node biopsies from patients with ALK^+ALCL were obtained from the Cross Cancer Institute (Edmonton, AB, Canada) following approval by the institutional ethics committee. The diagnosis of these cases was based on the criteria established by the World Health Organization Classification scheme, and all cases were confirmed to express ALK by immunohistochemistry. Immunohistochemical staining was performed using standard techniques. Briefly, formalin-fixed, paraffin-embedded tissue sections of 4 μM thickness were deparafinized and hydrated. Heat-induced epitope retrieval was performed using Tris buffer (pH 9.9; Dako) and a RHS. After incubation at 100°C for 10 minutes, slides were washed in running tap water for 5 minutes, and endogenous peroxidase were blocked using 10% H_2O_2 and methanol, followed by 5 minutes wash in running tap water. Tissue sections were then incubated with goat anti-human IL-21R (1:500) (Capralogics) or rabbit anti-human IL-21 antibody (1:500) (Capralogics) overnight in a humidified chamber at 4°C. Both antibodies are specific to peptide antigens and antigen sequence analysis indicates peptide antigen specificity. After 3 wash with PBS, tissue sections were incubated with secondary antibodies: anti-rabbit IgG or anti-goat IgG (Dako) for 30 minutes at room temperature. The tissue sections were incubated with 3,3'-diaminobenzidine/H_2O_2 (Dako) for color development, using hematoxylin as a counterstain. To optimize the IHC staining, benign tonsils were used as positive and negative controls for IL-21 in which the mantle zone B-cells served as negative control. For IL-21R, benign tonsils were used as a positive control and lung sample were used as a negative control.

4.2.10. Statistical Analysis

Graphpad Instat 3 software was used for statistical analysis of the data. The association between IL-22 and cell growth was evaluated using the Student's t-test. A *P* value <0.05 was considered statistically significant. Each assay was performed at least in triplicate and the illustrative graphs are representative of the mean of the triplicate experiments. Error bars represent SEM.

4.3. Results

4.3.1. Expression of IL-21 and IL-21R in ALK$^+$ALCL cell lines and tumors

The expression of IL-21 and IL-21R mRNA in three ALK$^+$ALCL cell lines was assessed using conventional RT-PCR studies. As shown in Figure 4.1, IL-21 mRNA was readily detectable in Karpas 299 but not in SU-DHL-1 and SUP-M2. In contrast, all three cell lines expressed *IL-21R*. The expression of γ_c in these cells has been previously reported by our group (Qiu et al., 2006). HepG2 cells served as the positive control and MDA-MB-231 served as the negative control for IL-21R.

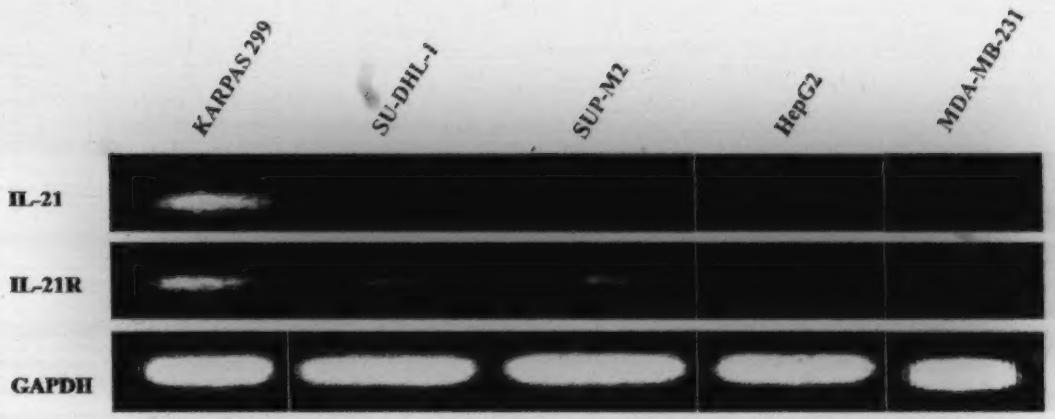

Figure 4.1. mRNA expression of IL-21 and IL-21R in ALK$^+$ALCL cell lines. RNA extraction was performed on ALK$^+$ALCL cell lines, SU-DHL-1, Karpas 299, SUP-M2 cells, and hepatocellular carcinoma cell line HepG2. RT-PCR studies demonstrated the presence of IL-21 mRNA in 1/3 ALK$^+$ALCL cell lines (expressed in Karpas 299). IL-21R mRNA was present in all 3 ALK$^+$ALCL cell lines. HepG2 was included as a positive control for IL-21R and MDA-MB-231 was included as a negative control for IL-21R. GAPDH was included as an internal control.

To further confirm the expression of IL-21R in ALK$^+$ALCL, Western blot analysis was performed on SU-DHL-1 and Karpas 299 cells. IL-21R protein expression was detected in both cell lines as well as HepG2 cells, which was included as a positive control (Figure 4.2). To determine the sub-cellular localization of IL-21R in Karpas 299 cells, immunofluorescence staining and confocal microscopy was performed. As shown in Figure 4.3, IL-21R was found on the cell membrane and in the cytoplasm of Karpas 299 cells. In contrast, the negative control using IgG antibody revealed no immunofluorescence staining.

To evaluate the expression of IL-21 and IL-21R mRNA in ALK$^+$ALCL tumors, RT-PCR was performed using frozen tumor tissues. All of these four tumors were previously confirmed to contain largely neoplastic cells by histologic examination. As shown in Figure 4.4, all 4 tumors had detectable IL-21 and IL-21R; while the IL-21R expression levels were relatively equal among all 4 tumors, the IL-21 level was appreciably lower in tumor 1 and 2 compared to that of tumor 3 and 4. HepG2 cells served as the positive control for IL-21R. MDA-MB-231 served as the negative control for IL-21R; both of these cell lines were negative for IL-21.

To confirm that the expression of IL-21R and IL-21 was indeed derived from the neoplastic lymphoid cells, immunohistochemistry was employed on formalin-fixed and paraffin-embedded tissues of ten ALK$^+$ALCL tumors. The staining for IL-21 is illustrated in Figure 4.5 (left panel). The staining was readily detectable in all

ALK^+ALCL cases. The neoplastic cells showed relatively intense cytoplasmic staining; the adjacent B-cell areas had no definitive IL-21 staining. For IL-21R, we were able to detect staining in the neoplastic cells in all 10 cases, as illustrated in Figure 1D (right panel), the neoplastic cells showed a cytoplasmic/membraneous staining pattern of IL-21R. The adjacent benign B-cell areas had no detectable IL-21R. IL-21 and IL-21R staining in reactive tonsils was also assessed; all have lymphoid cell compartments showed no definitive staining using immunohistochemical technique, strongly suggesting that both IL-21 and IL-21R are expressed at substantially higher levels in ALK^+ALCL when compared to benign lymphoid cells.

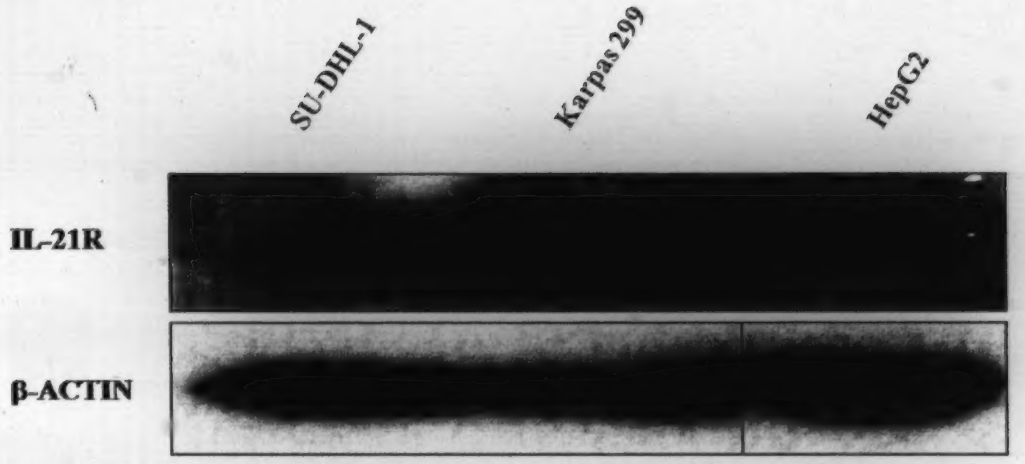

Figure 4.2. Protein expression of IL-21R in ALK$^+$ALCL. Protein lysates for SU-DHL-1, Karpas 299, and HepG2 were used and lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. IL-21R was detected using an anti-IL-21R antibody. Western blot analysis confirmed the expression of IL-21R in both ALK$^+$ALCL cell lines examined. HepG2 was included as a positive control. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

Figure 4.3. IL-21R expression in ALK$^+$ALCL cell lines. Immunofluorescence staining and confocal microscropy was employed to deteet IL-21R expression on ALK$^+$ALCL cell line Karpas 299. Karpas 299 cells were fixed, permeabilized, and stained with Alexa 488 to detect IL-21R expression. Immunofluorescence staining of slides with antibodies directed against IL-21R (left panel) or control IgG (right panel) and confocal microscopy demonstrated the expression of IL-21R in Karpas 299. Images were analyzed at 40X objective.

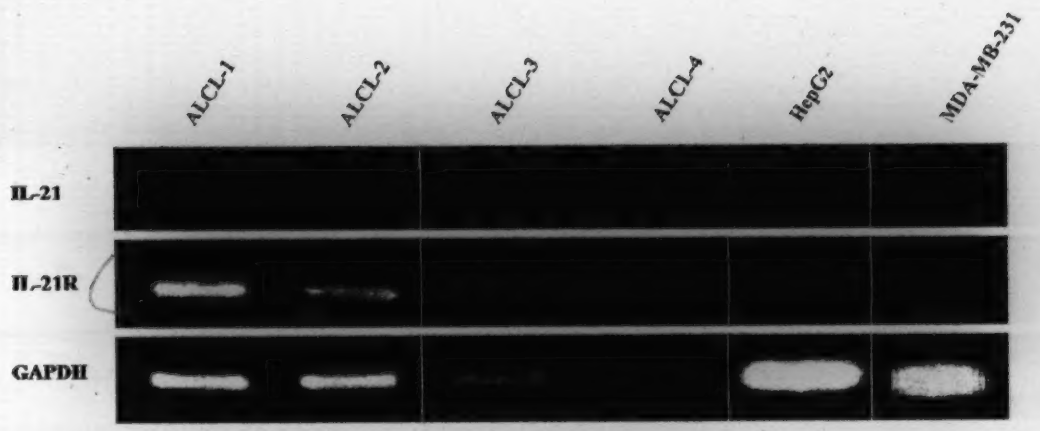

Figure 4.4. mRNA expression of IL-21 and IL-21R in ALK$^+$ALCL. RNA extraction was performed on 4 ALK$^+$ALCL tumors. RT-PCR studies demonstrated the presence of IL-21 and IL-21R mRNA in all 4 ALK$^+$ALCL tumors. IL-21R mRNA was present in all 3 ALK$^+$ALCL cell lines. HepG2 was included as a positive control for IL-21R and MDA-MB-231 was included as a negative control for IL-21R. GAPDH was included as an internal control.

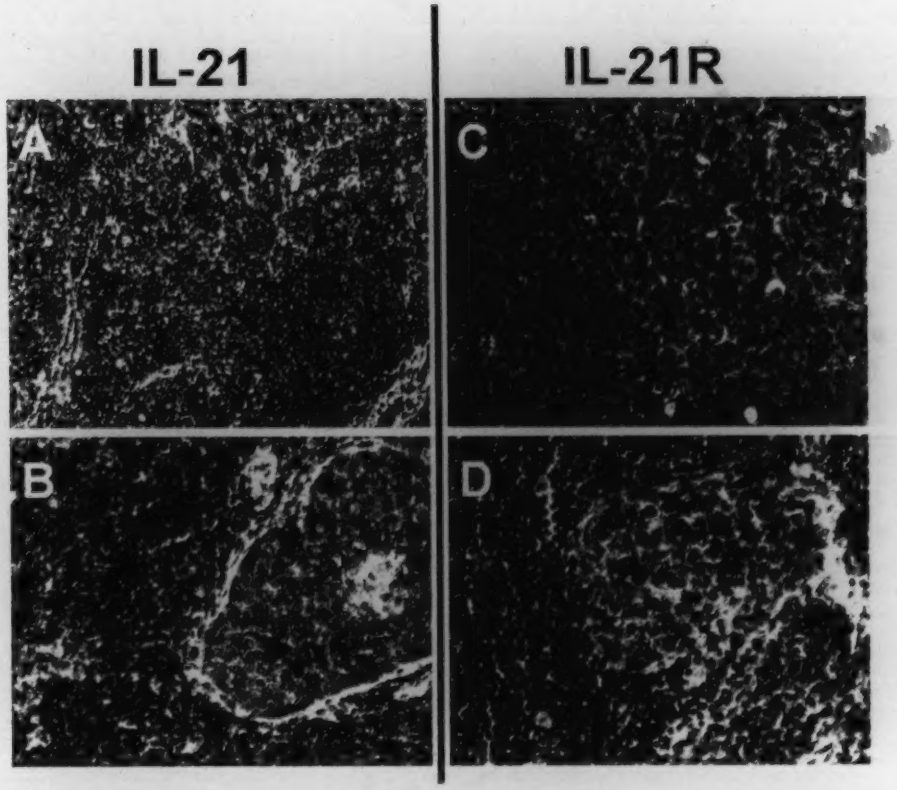

Figure 4.5. IL-21 and IL-21R expression in ALK^+ALCL tumors. Immunohistochemical staining of paraffin-embedded tissue sections confirmed the expression of IL-21 (left panel) and IL-21R (right panel) in a case of ALK^+ALCL tumors. A and C demonstrates staining at 20X magnification and B and D demonstrates staining at 40X magnification. For IL-21, the neoplastic cells showed cytoplasmic staining; the adjacent benign B-cell areas were largely non-reactive. IL-21R demonstrated a cytoplasmic/membranous staining pattern. The adjacent benign B-cell areas also were largely non-reactive.

4.3.2. IL-21 modulated JAK3/ STAT3 signaling

Since previous studies have reported a role for IL-21 in activating JAK3 and STAT3 (Brenne et al., 2002; de Totero et al., 2006; Habib et al., 2002; Pelletier et al., 2004; Ueda et al., 2005), I sought to determine whether IL-21 expression will contribute to the activation of these signaling pathways in ALK^+ALCL cells. Karpas 299 cells were serum-starved for 16 hours followed by treatment for 30 minutes with 10 ng/mL of recombinant IL-21 protein. As shown in Figure 4.6, IL-21 stimulation for 30 minutes led to an increase in pSTAT3 and pJAK3 expression. Next, I wanted to assess whether IL-21 will induce pSTAT1 activation, a member of the STAT family known to have anti-proliferative and pro-apoptotic properties (Adamkova et al., 2007; de Totero et al., 2006). Using the same experimental conditions, I demonstrated that STAT1 was not activated after IL-21 treatment. This is expected since an increase in cell growth, not apoptosis, was observed with treatment of recombinant IL-21 protein.

4.3.3. IL-21 treatment increased cell growth of ALK^+ALCL cell lines

To assess the biological effects of IL-21, I treated ALK^+ALCL cell lines with 10 ng/mL of recombinant IL-21 protein. SU-DHL-1 and Karpas 299 cells were grown in media containing reduced FBS (i.e. 5%) for 16 hours, followed by daily treatment with 10 ng/mL of rIL-21 for 5 days. Cell count was performed daily using the trypan blue exclusion assay. As shown in Figure 4.7, a significant increase in the number of viable cells was observed on day 3 for SU-DHL-1 and on day 4 for Karpas 299 cells. The delayed cell growth response in Karpas 299 is most likely due to the fact that Karpas 299, but not SU-DHL-1, produces endogenous IL-21. Morphologic examination of these cell

samples, either the negative controls or cells treated with rIL-21, did not show any features of apoptosis.

To further evaluate the biological role of IL-21 signaling in ALK^+ALCL, siRNA was employed to downregulate the expression of IL-21R in Karpas 299. RT-PCR was performed 24 hours after siRNA transfection and a substantial decrease in IL-21R was demonstrated in cells transfected with IL-21R siRNA but not control siRNA (Figure 4.8a). Correlating with these changes, pSTAT3 was substantially decreased in cells transfected with IL-21R siRNA compared to cells transfected with scrambled siRNA (Figure 4.8b). Using the same experimental conditions, I assessed if the cell growth was affected by IL-21R downregulation. Thus, I performed the MTS assay in cells transfected with IL-21R siRNA. At 72 hours after transfection, the cell growth index of cells transfected with IL-21R siRNA was significantly lower than that of the negative control sample (*P* value, 0.028) (Figure 4.8c).

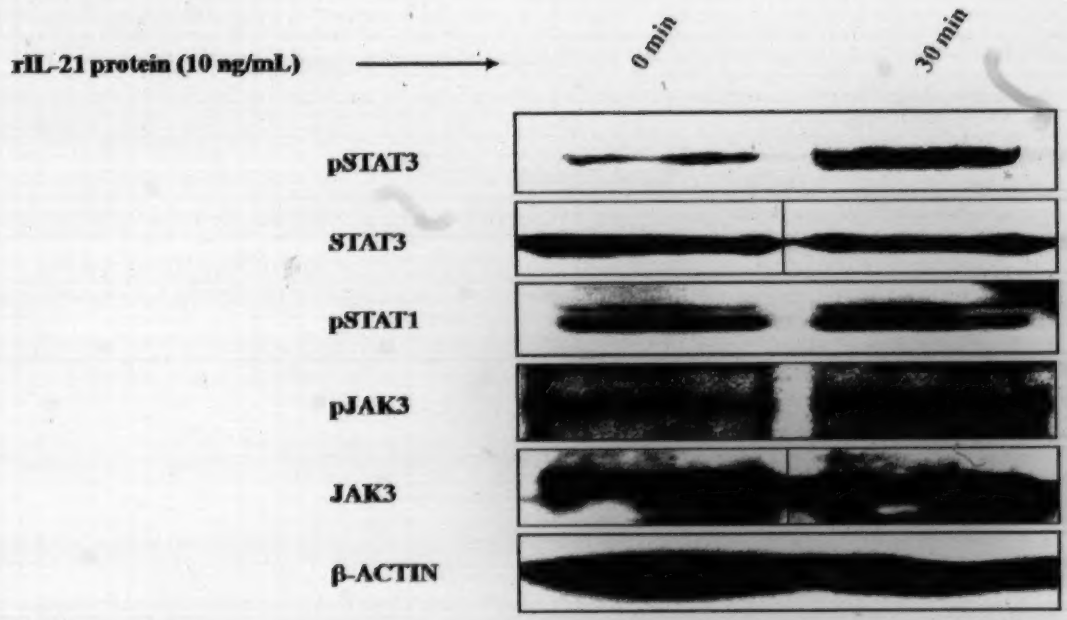

Figure 4.6. Effects of IL-21 on STAT3, JAK3, STAT1 signaling. Karpas 299 cells were exposed to 2 conditions: treatment with recombinant IL-21 protein (10 ng/mL) and no treatment. Karpas 299 cells were serum starved for 16 hours followed by treatment with 10 ng/mL of recombinant IL-21 protein for 0 and 30 minutes. Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. Phosphorylated STAT3, JAK3, ERK1/2, STAT1 was detected using anti-pSTAT3, anti-pJAK3, anti-pERK1/2, and anti-pSTAT1 antibody. This is followed by detection of the total cellular STAT3, JAK3, and ERK1/2 using anti-STAT3, anti-JAK3, and anti-ERK1/2 antibody. Treatment of Karpas 299 with 10 ng/mL of recombinant IL-21 protein for 30 minutes resulted in an increase in the phosphorylated forms of STAT3, JAK3, and ERK1/2 with the total levels of STAT3, JAK3, and ERK1/2 not changing between 0 and 30 minute treatments. Treatment of recombinant IL-21 protein for 30 minutes did not change the phosphorylated level of STAT1. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

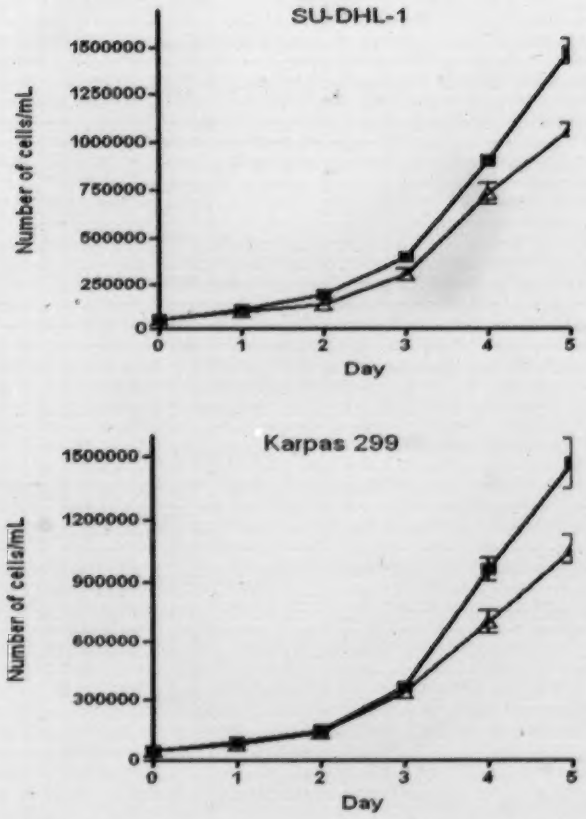

Figure 4.7. IL-21 modulated cell proliferation in ALK$^+$ALCL cell lines. 2 ALK$^+$ALCL cell lines were exposed to 2 conditions: treatment with recombinant IL-21 protein (■) or no treatment (△). SU-DHL-1 and Karpas 299 were serum starved for 16 hours followed by daily treatment with 10 ng/mL of recombinant IL-21 protein. Daily cell counts were performed using trypan blue exclusion assay. Cell numbers were counted using a hemocytometer at 10X objective. Treatment of 2 ALK$^+$ALCL cell lines with recombinant IL-21 protein induced a marked increase in cell number seen at approximately Day 4. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of the mean.

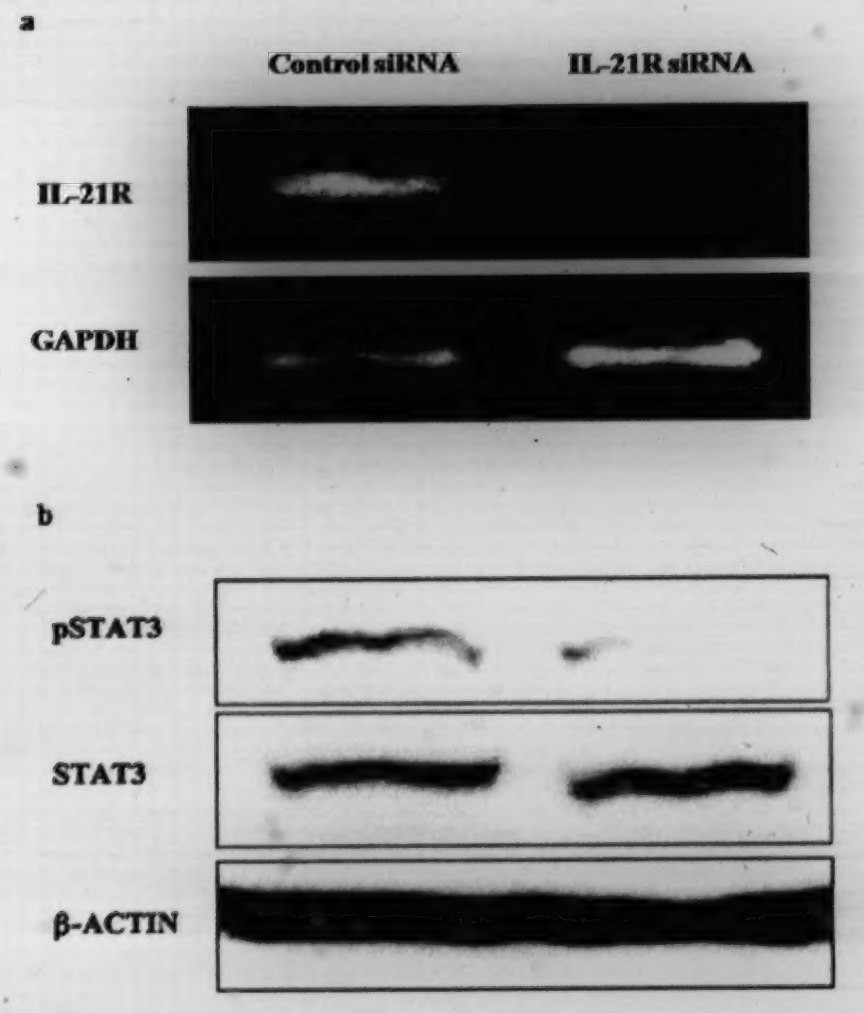

Figure 4.8. IL-21 directly up-regulated STAT3 and cell growth. Karpas 299 cells were transfected with either IL-21R siRNA or control siRNA. (a) After 24 hour incubation, RT-PCR studies confirmed the downregulation of IL-21R in the IL-21R siRNA sample compared to the control siRNA sample. (b) After 24 hour incubation, lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. Phosphorylated STAT3 was detected using anti-pSTAT3, antibody, followed by detection of the total cellular STAT3 using anti-STAT3 antibody. pSTAT3 expression was also downregulated in the IL-21R siRNA sample. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

c

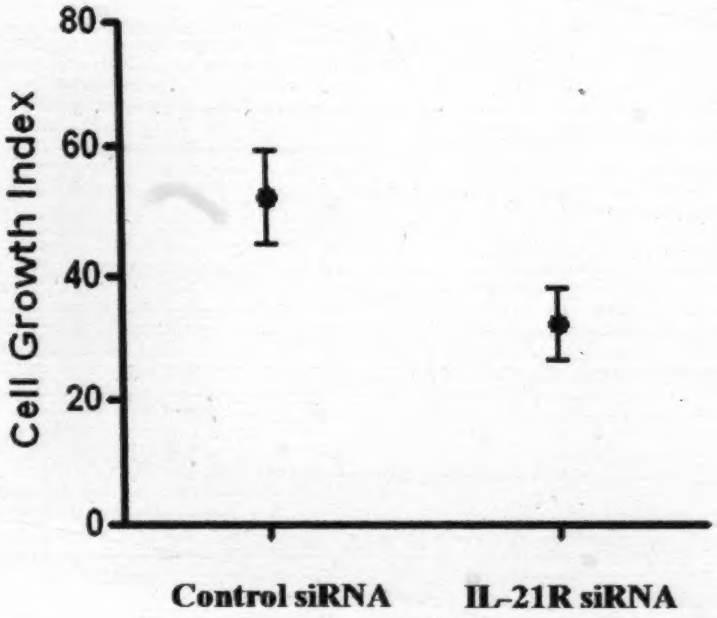

Figure 4.8. (continued) (c) After 72 hour incubation, cell viability was measured colorimetrically at 450 nm and absorbance values were normalized using the Microplate Manager 5.2.1. Transfection of Karpas 299 cells with IL-21R siRNA resulted in a significant decrease in cell growth compared to the control siRNA. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of the mean.

4.4. Discussion

The rationale for performing the study in this chapter is based on our previous finding that JAK3 is constitutively activated in ALK^+ALCL, and we believe that this finding is suggestive of a role of cytokine stimulation in the pathogenesis of these tumors. With this assumption, we started to investigate the possible role of various cytokines that normally activate JAK3 in these tumors. JAK3 is an interleukin receptor-bound tyrosine kinase in which activation is limited to a small number of interleukins that recruit the IL-2 common γ_c in their receptors. Thus, we have focused on those interleukins which signaling require γ_c recruited to their respective receptors, and they include IL-2, IL-9, IL-15, and IL-21 (Leonard, 2001; Russell et al., 1995). Previously, our group have described evidence to support the existence of the IL-9 autocrine stimulatory pathway in ALK^+ALCL. Specifically, blockade of IL-9 stimulation using a neutralizing antibody inhibits JAK3/STAT3 activation, accompanied by decreased cell growth and tumorigenicity in ALK^+ALCL cell lines (Qiu et al., 2006). In this chapter, I examined IL-21, a recently described type I cytokine produced exclusively by activated CD4-positive T cells. IL-21 has been described to have profound but heterogeneous biological effects in B-cells, T-cells and natural killer cells (Coquet et al., 2007; Habib et al., 2003; Kasaian et al., 2002; Parrish-Novak et al., 2000). Importantly, IL-21 is known to activate JAK3 in benign lymphoid cells (Parrish-Novak et al., 2000; Strengell et al., 2002). As described above, I was able to detect consistent expression of IL-21 and IL-21R in the cytokine oligonucleotide array studies of ALK^+ALCL cells. With this background, I

hypothesize that IL-21 is a contributing factor for JAK3/STAT3 activation and pathogenesis of ALK^+ALCL.

In keeping with this hypothesis, I confirmed the consistent expression of IL-21 in ALK^+ALCL cell lines and tumors. While evidence of IL-21R expression was only found in 1 of the 3 ALK^+ALCL cell lines examined, it was detectable in all 4 tumors by RT-PCR and all 10 tumors by immunohistochemistry. This data indicate that IL-21 signaling is biologically significant, since addition of recombinant IL-21 protein enhanced the activation of JAK3/STAT3, a major signaling pathway known to be important in ALK^+ALCL. Correlating with these biochemical changes, addition of recombinant IL-21 protein significantly increased cell growth and siRNA downregulation of IL-21R significantly decreased cell growth in ALK^+ALCL cell lines. In further support of the biological importance of the IL-21 signaling pathway in ALK^+ALCL, siRNA downregulation of IL-21R showed the opposite biological effects.

The tumor-promoting effects of IL-21 in ALK^+ALCL are in parallel with the observations made in myeloma (Brenne et al., 2002) and adult T-cell leukemia (Akamatsu et al., 2007): (Ueda et al., 2005). An increase in cell proliferation was observed in myeloma cells and T-cell leukemia cells when treated with rIL-21 (Akamatsu et al., 2007; Brenne et al., 2002; Ueda et al., 2005). Analysis of the JAK/STAT signaling pathway was described to some extent in these papers. Brenne *et al.* reported phosphorylation of JAK1 and STAT3, but not STAT1, after treatment of myeloma cells with rIL-21 (Brenne et al., 2002). These findings are indeed in parallel with our findings

regarding STAT1 and STAT3 activation. Ueda *et al.* demonstrated STAT3 and STAT5 phoshoryation after rIL-21 treatment of T-cell leukemia cells (Ueda et al., 2005), but STAT1 phosphorylation was not investigated in this study. The biological significance of IL-21-mediated STAT1 will be further discussed below.

The data presented in this chapter further supports the concept that STAT3 activation in ALK^+ALCL is multi-factorial, a concept that was initially proposed by Zhang *et al.* (Zhang et al., 2002). This multifactorial dysregulation of STAT3 was initially proposed to be attributed to the presence of NPM-ALK, the aberrancy of PP2A, a tyrosine phosphatase known to inhibit STAT3 dephosphorylation, and the absence of PIAS3 (Zhang et al., 2002). Nevertheless, the contribution of JAK3, the physiologic activator of STAT3, was not included in this study. Our findings related to IL-9 and IL-21 in activating JAK3/STAT3 and enhancing tumor growth in ALK^+ALCL not only have further supported the 'multi-factorial STAT3 activation' concept, but also added a new dimension to this conceptual model. In Chapter 3, I have provided evidence that the tumorigenicity of ALK^+ALCL is promoted by IL-22 (*Leukemia*, in press). Unlike IL-9 and IL-21, IL-22-mediated activation of IL-22 is not dependent on γ_c or JAK3. Furthermore, the functional IL-22 receptor (IL-22R), which is composed of the IL-22R1 and IL-10R2 subunits, is not fully expressed on benign lymphoid cells. The data in chapter 3 suggest that the aberrant expression of IL-22R1 in ALK^+ALCL cells is directly linked to NPM-ALK, since transfection of NPM-ALK into cells resulted in the expression of IL-22R1, thus converting from an 'IL-22 un-responsive phenotype' to an 'IL-22 responsive phenotype'. Taken together, it is increasingly evident that, while NPM-

ALK mediates tumorigenesis in ALK^+ALCL by deregulation of multiple signaling pathways, aberrancies of cell signaling in these neoplastic cells can be attributed to increasing number of factors. Recent studies from our group highlights the importance of autocrine cytokine stimulation of the STAT3 pathway, which is crucial for NPM-ALK-mediated lymphogenesis (Chiarle et al., 2005; Crockett et al., 2004).

Aside from activating STAT3, IL-21 signaling also has been reported to result in activation of STAT1 in some cell types (de Totero et al., 2006; Habib et al., 2003; Zeng et al., 2007). In contrast to STAT3 which promotes cell survival, cell cycle progression in many cell types, STAT1 is known to have tumor suppressing properties, namely anti-proliferative and pro-apoptotic effects (Adamkova et al., 2007; de Totero et al., 2006). In view of the normal functions of STAT1, I believe that the lack of IL-21 induced upregulation of pSTAT3 in ALK^+ALCL is significant. As discussed above, myeloma cells also fail to demonstrate STAT1 activation upon IL-21 stimulation (Brenne et al., 2002). Taken together, it is tempting to speculate that the IL-21 induced cell growth is attributed to the imbalance between STAT1 and STAT3 activity. It will be of great interest to determine why STAT1 is not activated in ALK^+ALCL or myeloma in response to IL-21.

In conclusion, I have provided the first evidence that an autocrine IL-21 stimulatory pathway exists in ALK^+ALCL tumors. In parallel with IL-9, IL-21 signaling contributes to cell growth in ALK^+ALCL by enhancing JAK3/STAT3 activation and may be a potential therapeutic target for this type of cancer. These findings support the

pathogenetic importance of JAK3 in these tumors. In conjunction with the results of several previous studies, it becomes increasingly evident that STAT3 activation, considered to be one of the most crucial oncogenic factors in ALK^+ALCL, is multi-factorial.

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Chapter 5 ♦

STAT3 is a transcriptional factor regulating the gene expression of *SALL4*

A version of this chapter will be submitted for publication to *Oncogene*: **Jennifer Dien Bard**, Pascal Gelebart, Leah Young, Amin HM, Yupo Ma, Raymond Lai. STAT3 is a transcriptional factor regulating the gene expression of *SALL4*.

5.1. Introduction

Signal transducers and activators of transcription (STATs) consist of 7 latent cytoplasmic transcription factors that are involved in cell cycle progression, differentiation and survival in response to various cytokines and growth factors (Clevenger, 2004; Yu and Jove, 2004). In contrast to normal cells in which phosphorylation and activation of STAT3 is a transient process, cancer cells often have constitutively active STAT3; aberrant STAT3 activation has been reported in several types of human cancers including breast cancer (Garcia et al., 2001), pancreatic cancer (Scholz et al., 2003) and lymphomas (Zamo et al., 2002). Using a variety of experimental models, it has been shown that constitutive activation of STAT3 directly contributes to oncogenesis (Bowman et al., 2000; Bromberg et al., 1999). Aside from its roles in oncogenesis, STAT3 has been implicated as a major player in murine embryonic development, as targeted disruption of the *STAT3* gene resulted in embryonic lethality (Takeda et al., 1997). STAT3 also has been strongly implicated in the maintenance of the pluripotent and self-renewal state in mouse embryonic stem (ES) cells, since blockade of STAT3 signaling using a dominant negative construct results in a loss in pluripotency and a transition into cell differentiation in these cells (Boeuf et al., 1997; Niwa et al., 1998; Raz et al., 1999).

The fundamental role of several other transcription factors in the self-renewal process of ES cells also has been established (Chambers and Smith, 2004; Niwa, 2001; Niwa et al., 2000; Vernallis et al., 1997). One of these proteins is SALL4, a zinc finger transcriptional factor that is one of four members of the SALL gene family originally cloned based on the

sequence homolog to *Drosophila spalt* (Al-Baradie et al., 2002; Kohlhase et al., 1996). SALL4 has been reported to play a prominent role in maintaining embryonic pluripotency, as knockdown of *SALL4* resulted in differentiation of ES cells (Ng et al., 2006). Similar to STAT3, SALL4 has been implicated in tumorigenesis in humans and is denoted a proto-oncogene (Al-Baradie et al., 2002; Ma et al., 2006; Yang et al., 2007). Ma *et al.* described the oncogenic role of SALL4 by reporting its constitutive activation in all cases of acute myeloid leukemia examined (Ma et al., 2006). In addition, *SALL4* mRNA was found expressed in human epithelial ovarian cancer, a cancer also known to have high activation of STAT3 (Bohm et al., 2006; Burke et al., 2001).

Since STAT3 and SALL4 share a similar role in maintaining the pluripotent state of the ES cells, these two proteins may be functionally linked to each other. In this study, I specifically tested the hypothesis that STAT3 is a transcriptional factor regulating the gene expression of *SALL4* using the tet-off STAT3C system previously established in Chapter 2 (Dien et al., 2006). MCF-7 cells permanently expressing tTA-TRE-STAT3C allows the level of STAT3 to be tightly controlled by the addition of tetracycline (STAT3C $^{\text{tet-off}}$ MCF-7). I provided evidence that STAT3 binds to the promoter of the SALL4 gene and significantly modulates the transcriptional activity of *SALL4*. The data presented in this chapter provides a novel connection between STAT3 and SALL4 in oncogenesis and a mechanism where constitutive activation of STAT3 can directly alter SALL4 expression.

5.2. Materials and Methods

5.2.1. Construction of tet-off STAT3C

STAT3C construct is a generous gift from Dr. Bromberg, and its properties have been well characterized and described (Bromberg et al., 1999). Briefly, the constitutively dimerizable form of STAT3 was created by substituting cysteine residues for specific amino acids within the C-terminal loop of the SH2 domain, allowing for sulfhydryl bonds to form between the STAT3 monomers and rendering the molecule capable of spontaneous dimerization without phosphorylation at Tyr705. Thus, the STAT3C dimer is capable of translocating to the nucleus and driving gene transcription. Due to its ability to transform cells and promote tumor formation, STAT3C has been widely used to alleviate the importance of STAT3 activation in human cancers. To create tet-off STAT3C, we first synthesized TRE-STAT3C, which was generated by digesting the 2.8 Kb fragment of STAT3C from the STAT3C plasmid using *Bam*H1. The STAT3C fragment was then ligated with the pTREzhyg vector (Clontech), which was then transformed into *E.coli* and selected on ampicillin resistant plates. The plasmids were isolated using the mini-prep kit (Qiagen). Orientation and integrity of the generated TRE-STAT3C plasmid was analyzed by DNA sequencing using the 3130XL Genetics Analyzer (Applied Biosystems). The TRE-STAT3C plasmid was amplified using the Maxiprep kit (Qiagen).

5.2.2. SALL4 Promoter Construct

The 5'-flanking region of SALL4 was amplified using the following primer set: 5'-GGGGTACCGCTCAATCAATTATTATTATTAC; 3'- CCCAAGCTTGCGAGCATCG GGGCGCCGGGAGAG to generate a fragment of 2000 base pairs upstream of the first ATG carrying Kpn and HindII restriction enzyme sites at each end, respectively. Human genomic DNA isolated from the kidney was used to amplify this 5'-flanking region of SALL4. After Kpn and HindII digestion, this fragment was cloned into the promoterless pGL3-Basic luciferase reporter plasmid (Promega, Madison, WI, USA) to generate the SALL4/luciferase reporter.

5.2.3. Cell lines and Culture

Human breast cancer cell lines MCF-7 (ATCC HTB-22), MDA-MB-231 (ATCC HTB-26) and MDA-MB-468 (ATCC HTB-132) were used in this study. AML cell line HL-60 (ATCC CCL-240) and MCL cell line Mino were included as controls. Mino cells were established in our lab and the characteristics were previously described (Amin et al., 2003a). Briefly, Mino cells have the mature B-cell immunophenotype, carry the t(11;14)(q13;q32), and express cyclin D1. All cell lines were grown at 37°C in 5% CO_2 and maintained in either DMEM or RPMI-1640 (Sigma-Aldrich). Culture media were enriched with 10% FBS (Gibco) and antibiotics (10,000 units/mL penicillin G, 10,000 µg/mL streptomycin, Gibco). The creation of permanent MCF-7 cell clones was previously described in Chapter 2. Briefly, MCF-7 was transfected with tTA plasmid (Clontech) and TRE-STAT3C plasmid followed by selection of cell clones expressing tTA-TRE-STAT3C using G418 (800 µg/mL). These cells are referred to as STAT3C $^{tet-}$

^{off} MCF-7. In contrast to the cell clone that was used in chapter 2, this STAT3C $^{tet\text{-}off}$ MCF-7 permanently expressed TRE-STAT3C.

5.2.4. Transfection

Transient transfection of cell lines, with various vectors was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cell lines were grown in 6-well culture plates (Bectin Dickinson Labware) without antibiotics. When cells reached approximately 90% confluency culture media was replaced with serum-free Opti-MEM I (Gibco), and the cells were transfected with DNA:Lipofectamine complex. For all of the *in vitro* experiments, MCF-7 cell clone permanently transfected with tTA-TRE-STAT3C (STAT3C $^{tet\text{-}off}$ MCF-7) were subjected to transiently transfection with 2 μg of SALL4 promoter luciferase (A gift from Dr. Yupo Ma), and 1 μg of β-galactosidase (β-GAL). To regulate the expression of STAT3C in these cells, various concentrations of tetracycline (ng/mL) (Invitrogen) were added to the cell culture.

5.2.5. Western Blot Analysis and Antibodies

Western blot analysis was performed using standard techniques. Briefly, 24 hours after transfection, cells were harvested by gentle scraping and washed twice in PBS (Sigma). Lysis buffer (20 mM tris-HCl, pH 7.5, 5.0 mM EDTA, 40.0 μg/mL leupeptin, 1 μM pepstatin, 1 mM 4-AEBSF) was added to the cell pellet for 30 minutes on ice followed by centrifugation at 14,000*g* for 10 minute at 4°C. The supernatant was removed and 50-100 μg of protein was run on SDS-PAGE. Protein quantification was carried out using

the Bradford assay according to the manufacturer's protocol (Biorad). After the proteins were transferred to nitrocellulose membranes, the membranes were blocked with 5% milk in TBS (Sigma) and 0.05% Tween-20 (Calbiochem), and then incubated with primary antibodies overnight at 4°C by 1 hour incubation followed by 1 hour incubation with horseradish peroxidase-conjugated secondary antibody (1:10000) (Jackson Immunoresearch Laboratories). The membranes were washed in TBS with 0.05% Tween-20 for 30 minutes between steps. Proteins were detected using the enhanced chemiluminescence detection kit (Amersham Life Sciences). The number in parenthesis after each antibody listed indicates the dilution at which it was used. Antibodies used included: mouse monoclonal anti-human FLAG (1:3000, Sigma-Aldrich), rabbit polyclonal anti-human STAT3 (1:500, Santa Cruz), mouse monoclonal anti-human PSTAT3 (1:500, Santa Cruz), rabbit polyclonal anti-human SALL4 (a gift from Dr. Yupo Ma), mouse monoclonal anti-human α-tubulin (1:1000, Santa Cruz) and mouse monoclonal anti-human β-actin (1:3000, Sigma-Aldrich). The generation of the SALL4 antibody was previously described (Ma et al., 2006). Briefly, to prepare an anti-peptide antibody, the peptide MSRRKQAKPQHIN of human SALL4 was chosen for its potential antigenicity (amino acids 1-13). SALL4 anti-peptide antibody was produced in rabbits in collaboration with Lampire Biological Laboratories (Piperville, PA).

5.2.6. Nuclear Extraction

Nuclear extraction of MDA-MB-231, MCF-7, SU-DHL-1, and Mino cells were performed using the Nuclear Extraction Kit according to the manufacturer's protocol for adherent and suspension cells (Panomics, Fremont, CA, USA). Briefly, Buffer A

Working Reagent (Buffer A, dithiothreitol (DTT), Protease inhibitor, Phosphatase inhibitor I/II) was added to 1 X 10^6 cells, rocked on ice for 10 minutes at 200 rpm, and centrifuged at 14,000 *g* for 3 minutes at 4°C. After the supernatant is removed (cytoplasmic portion), Buffer B Working Reagent (Buffer B, DTT, Protease Inhibitor Cocktail, Phosphatase Inhibitor I/II) is added to each cell pellet and rocked on iced for 2 hours at 200 rpm. Samples are centrifuged at 14,000 *g* for 3 minutes at 4°C and the supernatant (nuclear portion) is transferred to a new microcentrifuge tube for Western blot analysis.

5.2.7. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468, Mino, HL-60 and 9 primary breast tumors using TRIzol extraction according to the manufacturer's protocol (Gibco). RT was performed using 500 ng total RNA in a first strand cDNA synthesis reaction with superscript reverse transcriptase as recommended by the manufacturer (Invitrogen). GAPDH was included as an internal control. The SALL4 primer sequences were obtained from a previous paper by Cui *et al.* (Cui et al., 2006). Primer pairs were designed by Primer3 Input 0.4.0 to detect GAPDH. The primer sequences are as follow: SALL4 (500 bp) forward: 5′-CATGATGGCTTCCT TAGATGCCCCAG-3′, reverse: 5′-CCGTGTGTCATGTAGTGAACCTTTAAG-3′, GAPDH (316 bp) forward: 5′-AAGGTCATCCCTGAGCTGA-3', reverse: 5′-CCCTGTTGCTGTAGCCAAAT-3′. PCR was performed by adding 1 µL RT product into 25 µL volume reaction containing 1X buffer, 200 µM of each dNTPs, oligonucleotide primer, and 0.2 U Ampli*Taq* polymerase. For DNA amplification,

cDNA was denatured at 94°C for 1 min, subjected to primer annealing at 60°C for 1 min, followed by DNA extension at 72°C for 1 min for 40 cycles in a thermal cycler (Applied Biosystems). Amplified product were analyzed by DNA gel electrophoresis in 1% agarose and visualized by the Alpha Imager 3400 (Alpha Innotech). Sequencing of PCR product for SALL4 was performed using TOPO TA Cloning Kit (Invitrogen) and the 3130XL Genetics Analyzer (Applied Biosystems).

5.2.8. Luciferase Assay

Luciferase assay is a sensitive method of detecting firefly luciferase (*Photinus pyralis*) in mammalian cells using a FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany). Firefly luciferase protein catalyzes luciferin (Sigma) oxidation which results in light generation. STAT3C $^{tet\text{-}off}$ MCF-7 undergoes double transfection with pGL3 SALL4 reporter construct (a gift from Dr. Yupo Ma), and β-GAL using Lipofectamine 2000 (Invitrogen). Lysates were prepared 24 hour post-transfection on ice using freshly prepared lysis buffer (1 M Tris-HCL pH 7.8, 10% NP40, 1M DTT), incubated on ice for 15 minutes, and plated onto 96-well plate (Corning Life Sciences, Lowell, MA, USA). Samples were analyzed using the FLUOstar Optima microplate reader (BMG Labtech). The luciferase assay buffer is prepared fresh and consist of: 20mM tricine, 100 mM magnesium carbonate ($MgCO_3$), 1 M magnesium sulphate (MgSO4), 50 mM ethylenediaminetetraacetic acid (EDTA), 1 M DTT, 10 mM coenzyme A, 100 mM luciferin, and 100 mM ATP. A portion of the lysate is used to measure β-GAL, an internal control for transfection efficiency. 20 µL of lysate is added to 96-well culture plate containing 80 µL of dH_20 and 20 µL of ortho-Nitrophenyl- β-galactoside (ONPG)

and the mixture is incubated at 37°C for 1 hour. β-GAL activity is measured colorimetrically at 405nm using a Microplate reader (Biorad). The luciferase activity is normalized against the β-GAL activity to minimize experimental variability caused by differences in cell viability or transfection efficiency.

5.2.9. Chromatin Immunoprecipitation Assay

Chromatin Immunoprecipitation (ChIP) was performed using the Magna ChIP Assay Kit according to the manufacturer's protocol (Upstate, Charlottesville, VA). This kit is optimized for immunoprecipitation of transcriptionally active chromatin from mammalian cells. Briefly, histones were cross-linked to DNA by adding formaldehyde to a final concentration of 1% and incubated for 10 minutes at room temperature. 10X glycine was then added to each dish to quench unreacted formaldehyde. Cells were washed twice with cold PBS containing 1X Protease Inhibitor Cocktail II, and gently scraped into 1.5 mL microcentrifuge tubes. After centrifuging cells at 800 *g* for 10 minute at 4°C, ChIP lysis buffer (1% SDS, 10mM EDTA, 50 mM Tris-HCL pH 8.0) plus 1X Protease Inhibitor Cocktail II are added and incubated on ice for 15 minutes. This is followed by sonication of chromatin to an average size of ~ 500bp and immunoprecipitated with protein A magnetic beads and either rabbit anti-human STAT3 antibody or normal IgG antibody overnight at 4°C. The protein A magnetic beads were then pelleted using a magnetic separator (Upstate) and the protein A bead-antibody/chromatin complex was washed using the provided buffers. Histone complex were then eluted with ChIP elution buffer and incubated at 65°C for 2 hours with shaking, followed by 10 minute incubation at 95°C. Beads were then separated using the

magnetic separator and supernatant was transferred to a new microcentrifuge tube. DNA was recovered using the DNA Purification spin columns provided and then used for PCR. Primer pairs were designed by Primer3 Input 0.4.0 to detect SALL4 promoter region containing putative STAT3-binding sites. The primer sequences are as follow: Primer #1 (209 bp) forward: 5'- GCCCAGAGCAGTTATGGAAA-3', reverse: 5'-ATTGA CACATGATGCCTGGA-3', Primer #2 (220 bp) forward: 5'- GATAGCTGGAGCAA GGATGG-3', reverse: 5' ATGAGCCCTGACAGCTGATT-3'. PCR was performed by adding 500 ng of DNA product into 25 µL volume reaction containing 1X buffer, 200 µM of each dNTPs, oligonucleotide primer, and 0.2 U Ampli*Taq* polymerase. For DNA amplification, DNA was denatured at 94°C for 30 second, subjected to primer annealing at 60°C for 30 second, followed by DNA extension at 72°C for 30 second for 32 cycles in a thermal cycler (Applied Biosystems). Amplified product were analyzed by DNA gel electrophoresis in 1% agarose and visualized by the Alpha Imager 3400 (Alpha Innotech).

5.2.10. Site Specific Mutagenesis

The human SALL4 promoter consists of approximately 2.0 Kb genomic DNA. Potential STAT3-binding sites in the SALL4 promoter was determined by searching the 2.0 Kb region for consensus STAT-binding sites, ($TTN_{(4-6)}AA$) and consensus STAT3-binding sites, TTMXXXDAA (D: A, G, T; M: A, C) (Qian et al., 2006). Four sites at positions -199, - 1229, -1270, -1316 upstream of the initiation site were selected and specifically mutated using the GeneEditor in vitro Site-Directed Mutagenesis system according to the manufacturer's protocol (Promega). Site-directed mutagenesis is a valuable tool to

evaluate the function of DNA and protein and the GeneEditor system uses antibiotic selection to obtain high frequency of mutants. Design of the mutagenic oligonucleotides was performed according to the manufacturer's protocol. In general, greater than 24-base oligonucleotide with mismatch located in the center is sufficient for 2 or more mismatches. To stabilize the annealing between the nucleotide and template DNA, the 3' end of the oligonucleotide should end with either a G or C nucleotide. Oligonucleotide sequences are listed below with mutations underlined and in lower case: -1316 from ATGCCCAGAGCAGTTATGGAAAGACCAATC to ATGCCCAGAGCAGgcgtGGA AAGACCAATC, -1270 from GATGATTTCATCATTCATTTAACATTTATTG to GATGATTTCATCAgcgtTTTAACATTTATTG, -1229 from TTGTGTAAAGCAGT TAGCCAAATTAAGTATAC to TTGTGTAAAGCAGgcgtCCAAATTAAGTATAC, and -199 from GATCAATGAGGGCTTATTTAAATGATCTC to GATCAATGAGG GCgcgTTTAAATGATCTC. pGL3 SALL3 reporter construct was the template used for the mutagenesis study. Mutants were screened by direct sequencing using the 3130XL Genetics Analyzer (Applied Biosystems). Plasmids were amplified using Maxi-prep kit (Qiagen).

5.2.11. STAT3DN Adenoviral Vector

The production and characteristics of AdSTAT3DN have been previously described (Kunisada et al., 1998). Briefly, the adenoviral vector was deleted at the E1A region. Using site-directed mutagenesis technique, the STAT3 cDNA was modified such that the Tyr705 residue was replaced by phenylalanine. Expression of STAT3 was driven by the

rabbit β-actin promoter and cytomegalovirus enhancer. The construct was epitope-tagged with FLAG (DYKDDDDK) (Kodak) at the N terminal.

5.2.12. Generation of SALL4 siRNA Retrovirus

The generation of SALL4 siRNA construct was previously described (Yang et al., 2007) Briefly, the Phoenix packaging cells (ATCC SD-3443) were grown at 37°C in 5% CO_2 and maintained in maintained in DMEM (Sigma). Recombinant retroviruses were produced by using the Phoenix packaging cell line that was transfected with the pSuper construct containing the control RNAi sequence or sequence directed against SALL4. The viral supernatant was collected 48 h after transfection and filtered through a 0.45-μm filter.

5.2.13. MTS Assay

To determine the effect of SALL4 on cell viability, MDA-MB-231 cells were infected with either SALL4 siRNA or empty vector retrovirus and the MTS assay (Promega) was performed after 48 hours. 1,000 cells were seeded in 96-well culture plates and cell viability was measured daily using a colorimetric Microplate reader (Biorad) at 450nm and absorbance values were normalized using the Microplate Manager 5.2.1 (Biorad).

5.3. Results

5.3.1 SALL4 gene promoter contains the STAT3 consensus sequence

As summarized in Table 5.1 and 5.2, sequence analysis of the -2 Kb *SALL4* promoter region in both humans and mice revealed 27 potential binding sites for STAT that is characterized by the consensus sequence, $TTN_{(4-6)}AA$ (Seidel et al., 1995). Among these sites, 4 in humans and 10 in mice contain TTMXXXDAA (D: A, G, T; M: A, C), the consensus sequence of STAT3 (Ehret et al., 2001; Qian et al., 2006). In humans, the 4 STAT3 potential binding sites begin at the following positions: -199, - 1229, -1270, and -1316, upstream of the ATG transcription initiation site.

Table 5.1. Potential STAT3-binding sites in human SALL4 promoter
* Specific STAT3-binding sites

Site number	Location relative to ATG	Consensus sites $TTN_{(4-6)}AA$	Site number	Location relative to ATG	Consensus sites $TTN_{(4-6)}AA$
1	-73	TTTCCCAA	15	-1092	TTTTCTGTAA
2	-124	TTGATAATAA	16	-1229	**TTAGCCAAA ***
3	-199	**TTATTTAAA ***	17	-1242	TTGTGTAAA
4	-255	TTTGTCACAA	18	-1270	**TTCATTTAA ***
5	-433	TTCTACGTAA	19	-1316	**TTATGGAAA ***
6	-442	TTCCAAAA	20	-1360	TTACAATGAA
7	-472	TTTGGAGCAA	21	-1512	TTGTCCAA
8	-576	TTGGGGGAA	22	-1582	TTCAACAA
9	-618	TTAATCAAAA	23	-1622	TTATACATAA
10	-722	TTTTGGAA	24	-1634	TTGCTTTAAA
11	-799	TTTGTGAA	25	-1655	TTGGCTATAA
12	-847	TTGAAGTTAA	26	-1691	TTATTAAA
13	-1046	TTAGTAACAA	27	-1879	TTTTCCTTAA
14	-1071	TTGCACAA			

Table 5.2. Potential STAT3-binding sites in mouse SALL4 promoter
* Specific STAT3-binding sites

Site number	Location relative to ATG	Consensus sites $TTN_{(4-6)}AA$	Site number	Location relative to ATG	Consensus sites $TTN_{(4-6)}AA$
1	-203	**TTATTTAAA ***	15	-1041	TTAGGAAA
2	-410	**TTCAATAAA ***	16	-1076	**TTGAGAAAA***
3	-439	TTCTAGAA	17	-1100	TTAGCCAA
4	-482	TTACTACAA	18	-1111	TTGCAAGGAA
5	-614	**TTCCGTAAA ***	19	-1137	TTCTTCAA
6	-683	TTAGGTAA	20	-1147	**TTAGTTGAA ***
7	-775	TTACAACAA	21	-1447	TTTCTGTAA
8	-794	**TTCCGAAAA***	22	-1478	TTGCAGAA
9	-856	TTCTAGCAA	23	-1692	**TTCCCAGAA ***
10	-869	TTCTGGAA	24	-1774	**TTCCCTTAA ***
11	-942	TTAAGTACAA	25	-1785	TTACACAA
12	-955	**TTCTAGAAA ***	26	-1808	**TTAGATAAA ***
13	-983	TTCTGTAA	27	-1919	TTATGAAA
14	-1024	TTCCCGAA			

5.3.2. STAT3 regulates *SALL4* gene expression

To establish the functional relationship between the STAT3 activity and *SALL4* gene expression, the *in vitro* model that was previously established and described in chapter 2 was employed. The expression of STAT3C in MCF-7 breast cancer cell line (STAT3C $^{tet\text{-}off}$ MCF-7) can be downregulated by the addition of tetracycline to the cell culture in a dose-dependent fashion (Dien et al., 2006). As shown in Figure 5.1a, the FLAG epitope that has been tagged to STAT3C construct was strongly expressed in the absence of tetracycline. With the addition of increasing doses of tetracycline to the cell culture (20 and 60 μg/mL), there was a gradual decrease in the expression of the FLAG epitope. Accordingly, the total STAT3 level, which included both the endogenous STAT3 and exogenous STAT3C, was also decreased. β-actin, the loading control, was unchanged in all 3 lanes. As shown in Figure 5.1b, which was derived from the same Western blots used for Figure 5.1a, the protein expression of SALL4 was significantly decreased with the addition of tetracycline; it was undetectable at 60 of μg/mL of tetracycline. HL-60 (an AML cell line) served as the positive control whereas Mino (a MCL cell line) served as the negative control for SALL4. To confirm these results, RT-PCR was performed and consistent results were obtained. As shown in Figure 5.2, STAT3C $^{tet\text{-}off}$ MCF-7 cells expressed *SALL4* mRNA at a much higher level in the absence of tetracycline treatment, compared to cells treated with tetracycline, at 20 or 60 μg/mL. HL-60 and Mino cell lines served as the positive and negative control, respectively. The sequences of the RT-PCR amplicons for SALL4 were confirmed (results not shown).

To further establish the functional relationship between the STAT3 activity and *SALL4* gene expression, STAT3C $^{tet\text{-}off}$ MCF-7 cells were transfected with a vector containing the human *SALL4* promoter/pGL3-basic luciferase reporter. All the transfection experiments performed included a β-GAL expressing vector as a control for the transfection efficiency. A relatively robust luciferase activity was detectable in the absence of tetracycline, and hence, a high level of STAT3C expression. As shown in Figure 5.3, addition of tetracycline resulted in a significant decrease in the *SALL4* promoter luciferase activity. The relative luciferase activity was calculated by normalizing the luciferase activity to the β-GAL activity.

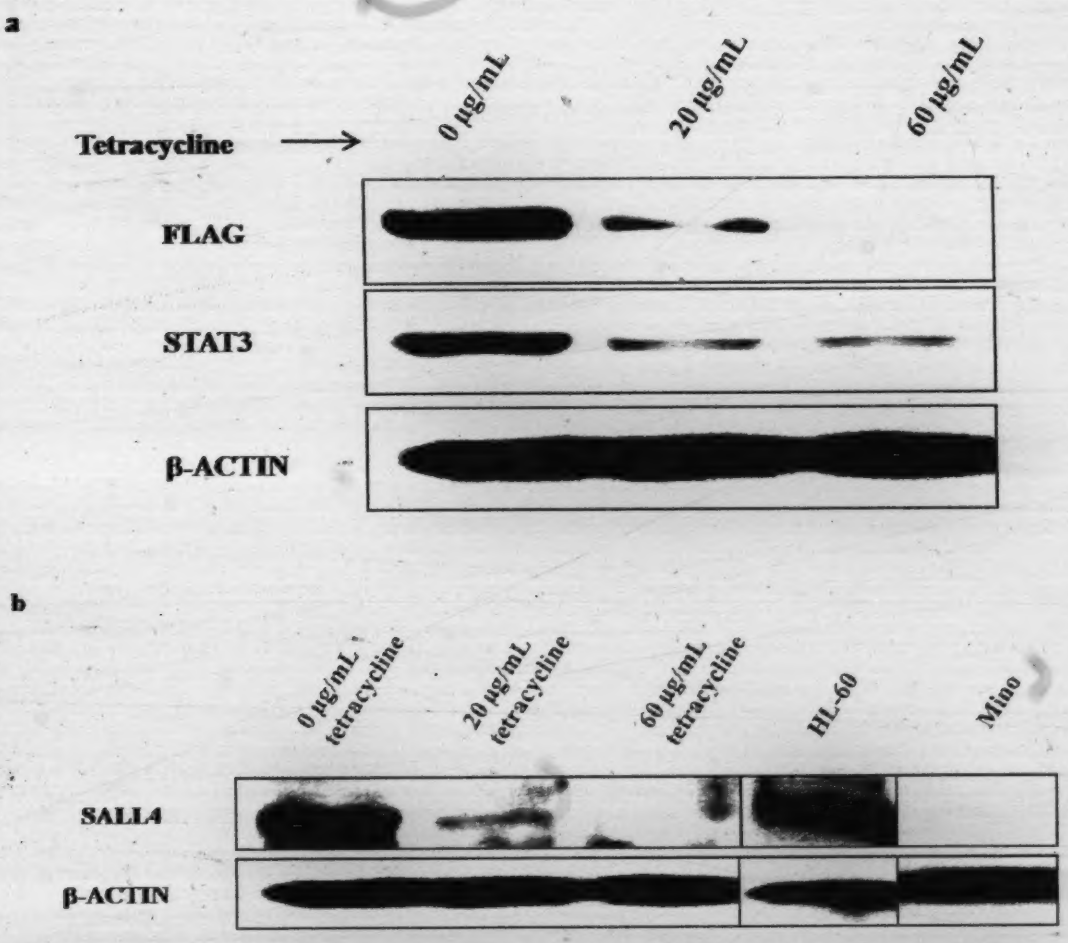

Figure 5.1. SALL4 expression in STAT3C $^{tet\text{-}off}$ system. MCF-7 cells permanently expressing tTA-TRE-STAT3C were treated with 0, 20, and 60 µg/mL of tetracycline and SALL4 expression was assessed by Western blot. (a). Flag, the epitope tagged in the STAT3C construct was detected using an anti-FLAG antibody. Detection of the total cellular STAT3 followed using an anti-STAT3 antibody. Treatment with increasing concentrations of tetracycline resulted in a decrease in FLAG and STAT3 expression with FLAG levels undetectable at 60 µg/mL. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control. (b). SALL4 expression was detected using an anti-SALL4 antibody. SALL4 expression was detected in the STAT3C $^{tet\text{-}off}$ MCF-7 cells that were not treated with tetracycline. In contrast, the addition of tetracycline was sufficient in shutting down SALL4 expression. AML cell line, HL-60, was included as a positive control for SALL4 and MCL cell line, Mino, were included as a negative control. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control.

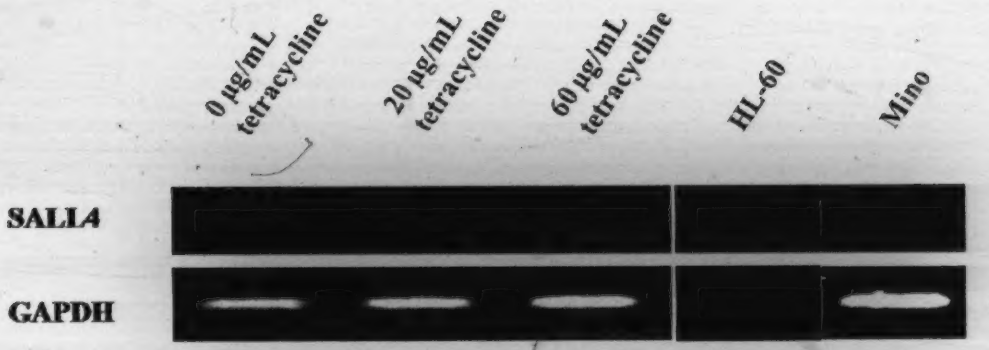

Figure 5.2. SALL4 mRNA expression STAT3C $^{tet\text{-}off}$ system. RNA extraction was performed on STAT3C $^{tet\text{-}off}$ MCF-7 cells treated with 0, 20, and 60 µg/mL of tetracycline. RT-PCR studies demonstrated the presence of SALL4 in the 0 and 20 µg/mL of tetracycline sample but SALL4 mRNA expression was absent in the 60 µg/mL of tetracycline sample. HL-60 was included as a positive control and Mino was included as a negative control for SALL4. GAPDH was included as an internal control.

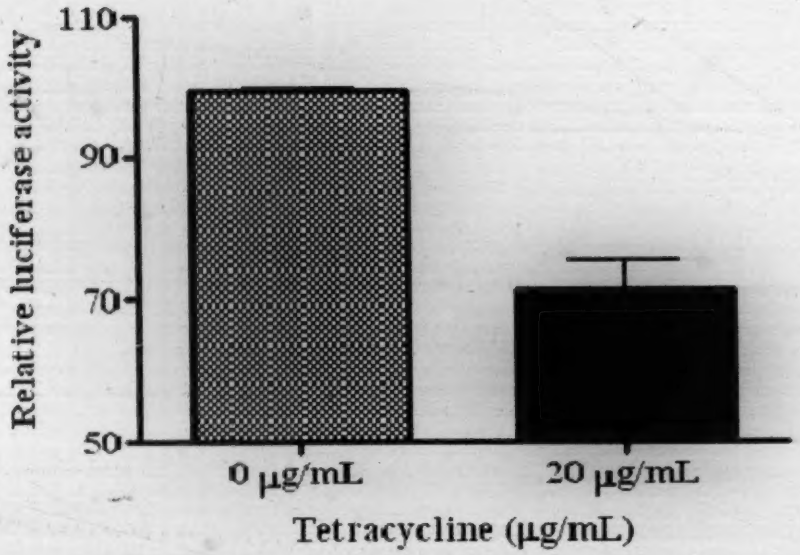

Figure 5.3. SALL4 transcriptional activation is STAT3 dependent. Luciferase assay was performed on STAT3C $^{tet\text{-}off}$ MCF-7 cells treated with 0 and 20 µg/mL of tetracycline. Cells were transiently transfected with human SALL4 luciferase promoter and β-GAL. Luciferase activity was read using a luminometer and levels were normalized with the β-GAL activity. Luciferase activity was much higher in the transfected cells with no tetracycline treatment compared to the transfected cells treated with 20 µg/mL of tetracycline. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of the mean.

5.3.3. STAT3 binds to *SALL4* Promoter

To determine whether there is a direct binding of STAT3 to the STAT3-binding consensus sequences on the *SALL4* promoter, chromatin immunoprecipitation was performed using MDA-MB-231, a breast cancer cell line expressing high activated STAT3. After extraction, chromatin was immunoprecipitated using anti-STAT3 antibody. Immunoprecipitation with normal rabbit IgG antibody was used as the negative control. Figure 5.4a is a schematic representation of the primer sets that were design to include the 4 putative STAT3-binding sites on the human *SALL4* promoter, with primer #1 spanning 3 STAT3 sites (-1316, -1270, -1229) and primer #2 spanning 1 STAT3 site (-199). The use of both primer #1 and primer #2 yielded amplifiable products of the *SALL4* promoter (Figure 5.4b). No amplifications were observed in the immunoprecipitation reactions that contained normal IgG antibody. The input lane was included as a control for PCR effectiveness. PCR without the addition of DNA templates was used as the negative control.

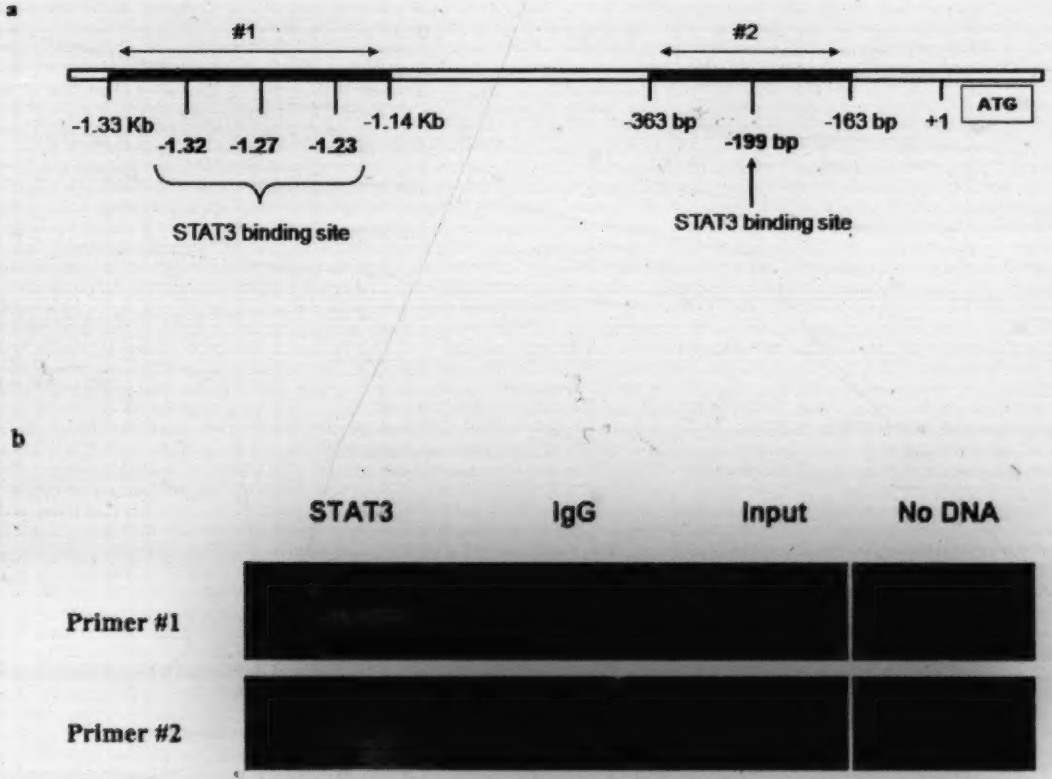

Figure 5.4. STAT3 binds to the human SALL4 promoter. (a). Schematic representation of the primer sets specific for the SALL4 promoter that was used in this study. Primer #1 consists of 3 STAT3-binding sites and primer #2 consist of 1 STAT3-binding site. (b). Chromatin immunoprecipitation was performed on breast cancer cell line MDA-MB-231, using antibody against STAT3 or normal rabbit IgG antibody. PCR was performed on the purified DNA using the 2 primer sets. PCR with primer #1 and #2 revealed amplicons for MDA-MB-231 using anti-STAT3 antibody. In contrast, no amplicons were observed in the anti-IgG antibody samples.

Site-directed mutagenesis was used to mutate all of the 4 putative STAT3-binding sites. Mutations were confirmed by DNA sequencing and are described as followed: -1316 from TTATGGAAA to gcgtGGAAA, -1270 from TTCATTTAA to gcgtTTTAA, -1229 from TTAGCCAAA to gcgtCCAAA, and -199 from TTATTTAAA to gcgTTTAAA. STAT3C $^{tet\text{-}off}$ MCF-7 cells were transfected with β-GAL, and with either wild type or the mutated constructs. Subsequently, luciferase assay was performed. After normalization with β-GAL, the relative luciferase activity for each mutated constructs was compared to that of the un-mutated construct. The wild type luciferase promoter demonstrated the highest level of luciferase activity. In contrast, mutations at the promoter region at -199 resulted in the most significant down-regulation of *SALL4* promoter activity (Figure 5.5). The luciferase activity was also significantly reduced in the other mutants. These results correlated with those found in the chromatin immunoprecipitation assay, supporting the concept that there are more than one functional STAT3-binding site on the *SALL4* promoter. Nevertheless, the STAT3-binding site at -199 appears to be most effective in modulating the gene expression of *SALL4*.

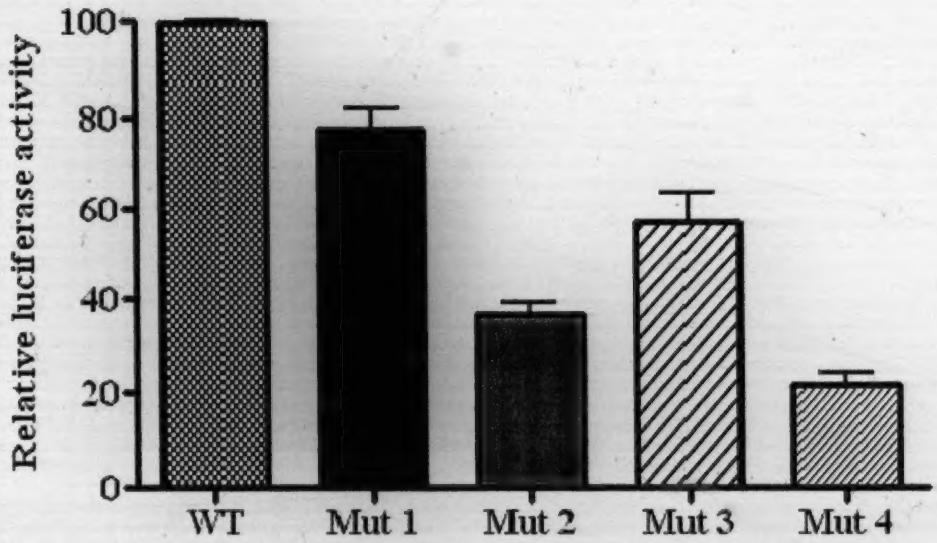

Figure 5.5. Transcriptional activation of *SALL4* promoter with altered STAT3-binding sites compared to wild type. Site-directed mutagenesis was performed on the *SALL4* promoter with mutations of 3-4 basepairs on the 4 putative STAT3-binding sites: -1316 (Mut 1), -1270 (Mut 2), -1229 (Mut 3), -199 (Mut 4). Transcriptional activity of the *SALL4* promoter was compared between the 4 mutants and the wild type. Constructs + β-GAL were transiently transfected in STAT3C $^{tet\text{-}off}$ MCF-7 cells with no tetracycline treatment and luciferase assay was performed. Luciferase activity was normalized against β-GAL activity. Luciferase activity of all mutants was decreased when compared to the wild type with the most significant decrease founding Mut 4 (-199). Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of the mean.

5.3.4. Detection of SALL4 in human cell lines

Since much of the data presented thus far in this chapter was based on the use of the *SALL4*/luciferase reporter gene construct, I tested if the SALL4 expression level can be regulated by modulating STAT3 activity in a native human cell line. While the expression of SALL4 is highly restricted in human cells (Cui et al., 2006), I was able to identify a relatively high level of SALL4 expression in several breast cancer cell lines, including MCF-7, MDA-MB-231, and MDA-MB-468, as shown in Figure 5.6a. HL-60 was included as the positive control and Mino cells were included as the negative control. To confirm *SALL4* expression in these cell lines, RT-PCR was performed and *SALL4* mRNA was detected in all three breast cancer cell lines (Figure 5.6b). The amplifiable PCR products were sequenced and the products were confirmed to be full-length SALL4 (data not shown). Since SALL4 is known to be a transcriptional factor itself, it is expected to be localized to the nucleus (Ma et al., 2006). Thus, nuclear-cytoplasmic fractionation was performed. As shown in Figure 5.7, I confirmed that SALL4 is localized to the nucleus in MDA-MB-231 and MCF-7 cells. Interestingly, the SALL4 nuclear protein level was higher in MDA-MB-231 cells, which correlated with a substantially higher pSTAT3 level in the nuclei of MB-231 cells. The same membrane was re-probed with an antibody reactive with α-tubulin, a protein largely restricted to the cytoplasm; the absence of α-tubulin in the nuclear fraction indicates that our fractionation method was sufficient.

Using MDA-MB-231, which expressed both SALL4 and STAT3, I determined if STAT3 regulates *SALL4* gene expression in a native condition. STAT3 activation was inhibited

with the use of an adenoviral STAT3 dominant negative construct (Ad-STAT3DN), which has been previously described in details (Khoury JD, 2003). As shown in Figure 5.8, MDA-MB-231 cells infected with Ad-STAT3DN for 24 hours showed expression of the FLAG epitope that was associated with the STAT3DN construct. *SALL4* expression was down-regulated in these cells. These results were in contrast with cells treated with an adenoviral vector expressing green fluorescence protein (Ad-GFP).

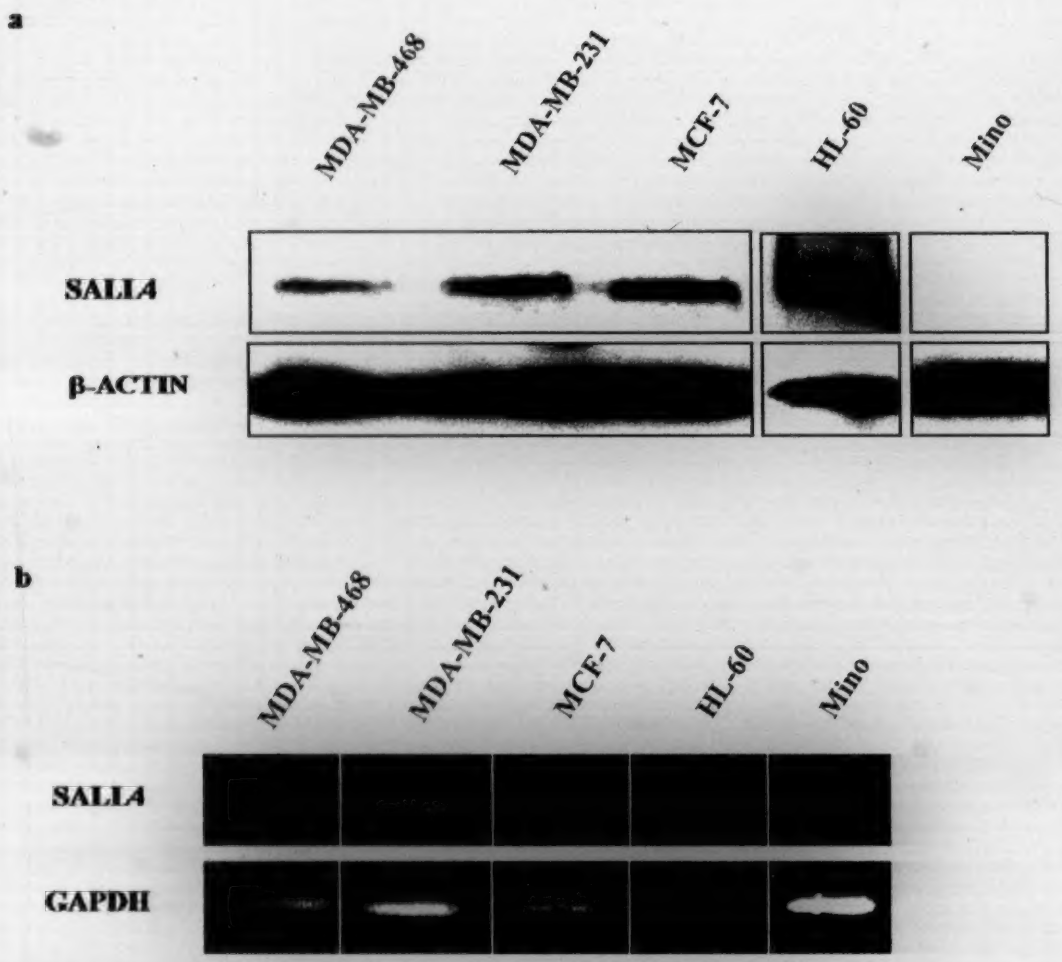

Figure 5.6. Expression of SALL4 in breast cancer cell lines. (a) Protein lysates for MDA-MB-468, MDA-MB-231, MCF-7, HL-60, and Mino were used and lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. SALL4 was detected using an anti-SALL4 antibody. Western blot analysis confirmed the expression of SALL4 in all breast cancer cells lines examined. HL-60 was included as a positive control and Mino were included as a negative control for SALL4. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control. (b) RNA extraction was performed on MDA-MB-468, MDA-MB-231, MCF-7, HL-60, and Mino. RT-PCR studies demonstrated the presence of SALL4 in breast cancer cell lines. HL-60 was included as a positive control and Mino was included as a negative control for SALL4. GAPDH was included as an internal control.

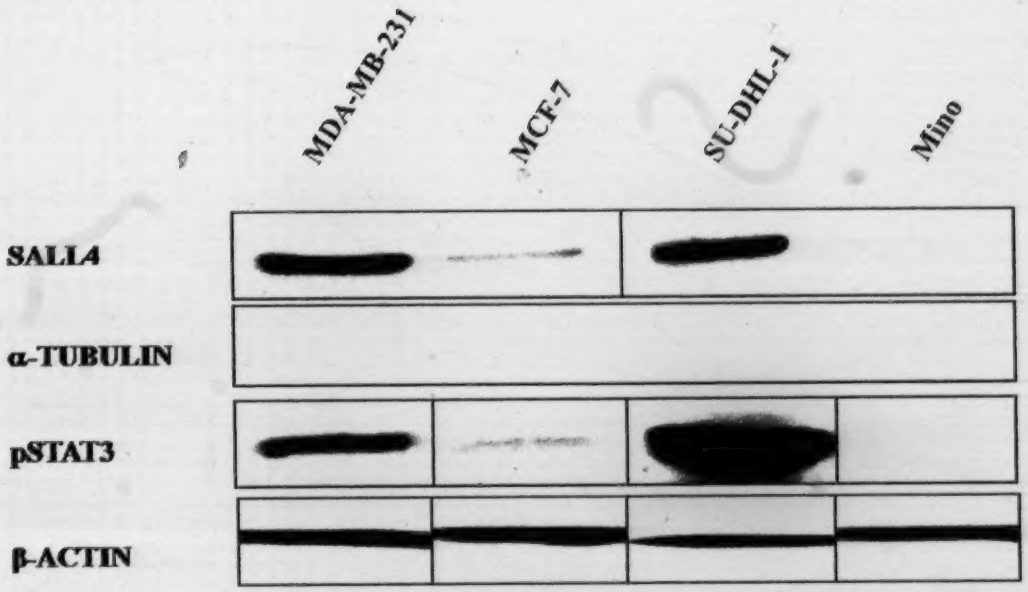

Figure 5.7. Nuclear SALL4 correlated with pSTAT3 in breast cancer. Nuclear extraction was performed on MDA-MB-231, MCF-7, SU-DHL-1, and Mino. Western blot analysis demonstrated the presence of nuclear SALL4 in both breast cancer cell lines and tumor. SALL4 was detected using an anti-SALL4 antibody and α-Tubulin, a cytoplasmic protein, was detected using anti- α-Tubulin antibody. This was included to ensure that no cytoplasmic protein was carried over during nuclear extraction. Levels of nuclear SALL4 expression was compared to pSTAT3 levels in these cell lines. Phosphorylated STAT3 was detected using anti-pSTAT3 antibody. Nuclear SALL4 expression correlated with pSTAT3 levels. ALK+ALCL cell line was included as a positive control and Mino was included as negative control for both SALL4 and pSTAT3. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control for pSTAT3.

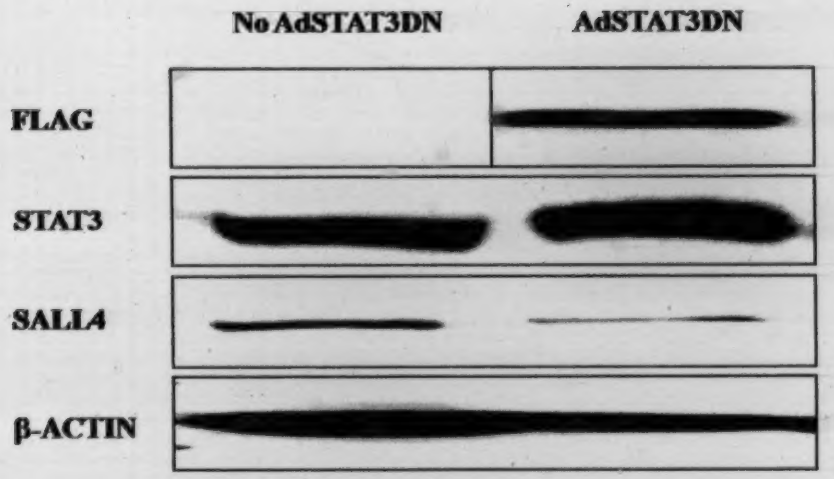

Figure 5.8. STAT3 up-regulated SALL4 expression. MDA-MB-231 cells were treated with AdSTAT3DN for 24 hours and protein lysates was generated. Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. Untreated cells were included as a negative control. In the AdSTAT3Dn treated cells, FLAG expression was detected and total STAT3 levels was increased and is representative of a decrease in phosphorylated STAT3. In contrast, SALL4 levels were decreased with the addition of AdSTAT3DN, indicating that STAT3 can activate SALL4 expression. FLAG antibody was detected using anti-FLAG antibody. Total STAT3 was detected using anti-STAT3 antibody and SALL4 was detected using an anti-SALL4 antibody. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control

5.3.5. Effects of endogenous SALL4 on human cancer cell lines

To assess if the expression of SALL4, which is under the regulation of STAT3, is biologically meaningful, SALL4 expression was down-regulated in MDA-MB-231 using siRNA-mediated knockdown. MDA-MB-231 was infected with either SALL4 siRNA retrovirus or an empty-vector retrovirus for 48 hours. Trypan blue exclusion assay was used to count the number of viable cells and to assess cell morphology. There was a significant decrease in the number of viable cells in cells treated with SALL4 siRNA, but no appreciable apoptosis was detectable. Western blots were performed to confirm that SALL4 expression was down-regulated with the siRNA treatment. As illustrated in Figure 5.9a, SALL4 expression was down-regulated by approximately 50%. To further assess the effects of SALL4 on cell growth, MTS assay was performed 48 hours after retroviral infection. The cell growth index was measured daily for 4 days and a significant decrease in cell growth was observed in the SALL4 siRNA infected cells when compared to cells infected with the empty vector (Figure 5.9b). On day 4, there was approximately a 50% decrease in cell growth in the SALL4 siRNA sample when compared to the control sample

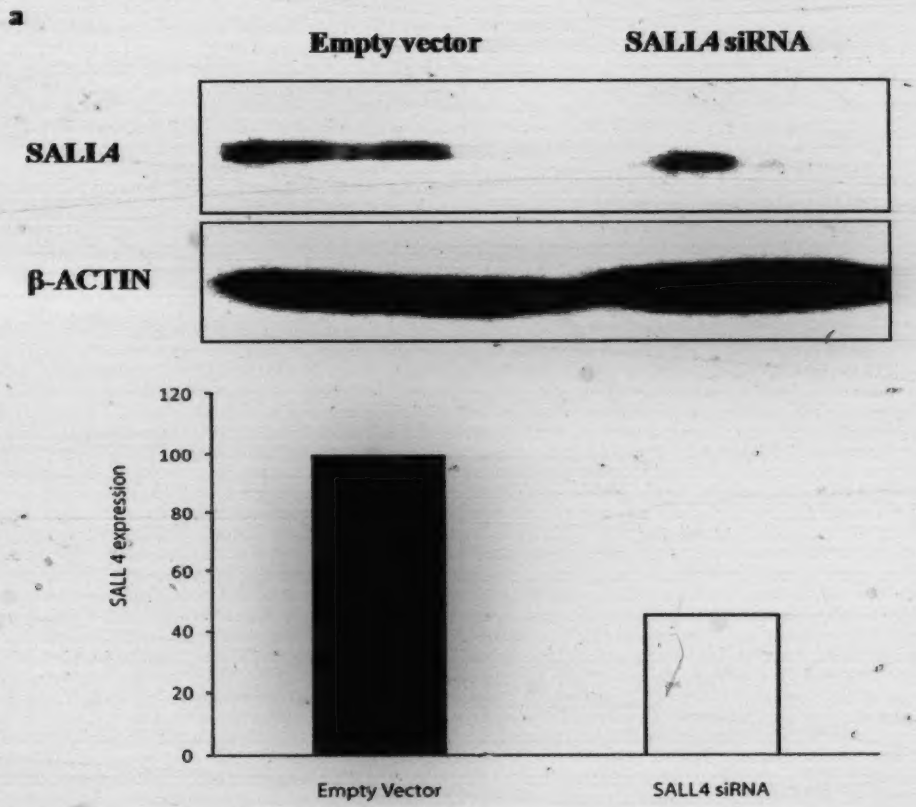

Figure 5.9. SALL4 promote cell growth in breast cancer. (a). MDA-MB-231 cells was treated with SALL4 siRNA retrovirus or empty vector and protein lysates was generated. Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. SALL4 levels were decreased on the siRNA treated cells compared to the empty vector treated cells. SALL4 was detected using an anti-SALL4 antibody. Nitrocellulose membrane stained for β-ACTIN was included as a protein loading control. Densitometry was performed to quantify the protein expression levels of SALL4.

b

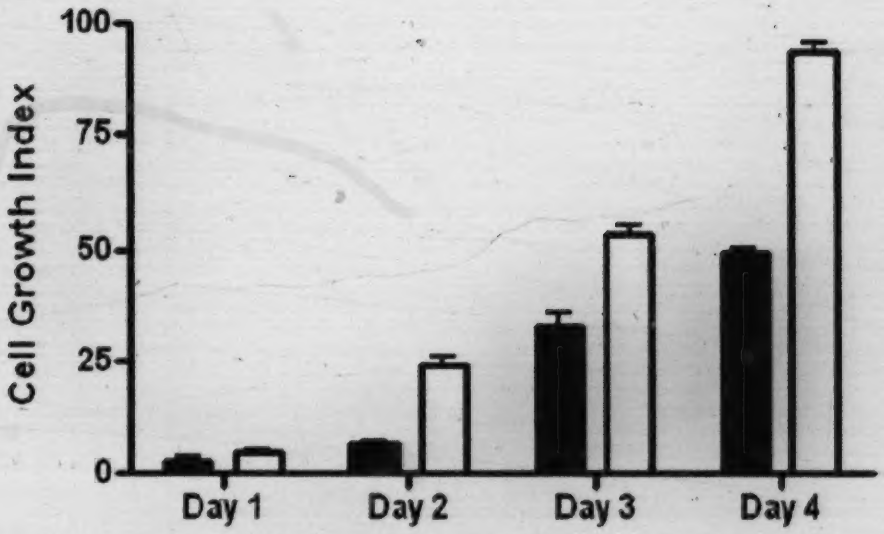

Figure 5.9. (continued) (b). MDA-MD-231 cells were treated with SALL4 siRNA retrovirus (■) or empty vector (□). After 48 hours, cells were plated onto a 96-well culture plate (1,000 cells/well). Cell viability was measured colorimetrically at 450 nm and absorbance values were normalized using the Microplate Manager 5.2.1. Treatment of MDA-MB-231 with SALL4 siRNA retrovirus resulted in a decrease in cell growth compared to cells treated with the empty vector. Cell growth index was approximately 50% less in SALL4 siRNA retrovirus treated cells at Day 4. Graphical depiction illustrates the mean of triplicate experiments. Error bars represent standard error of the mean.

5.4. Discussion

Since its discovery, STAT3 have been extensively studied, and it has been known to be critical for cell growth, differentiation and survival in normal cells. Aberrant activation of STAT3 is oncogenic and this biochemical abnormality is detectable in various cancer cell lines and tumors (Bowman et al., 2000; Calo et al., 2003; Levy and Lee, 2002). STAT3 is also known to play a critical role in maintaining the pluripotency and self-renewal state of mouse ES cells (Boeuf et al., 1997; Niwa et al., 1998). Murine ES cell lines are pluripotent cell lines derived from the inner cell mass (Evans and Kaufman, 1981; Martin, 1981) and its self-renewal is dependent on LIF, a cytokine known to mediate cellular response via heterodimerization of LIF receptor and gp130. The binding of LIF to the receptor complex triggers the activation of JAK tyrosine kinases, resulting in STAT3 activation (Gearing et al., 1991; Smith et al., 1988).

Similar to STAT3, SALL4, a newly designated proto-oncogene, also plays a fundamental role in the self-renewal and pluripotency of ES cells (Zhang et al., 2006). *SALL4* gene is one of four family members originally cloned on the basis of its DNA sequence homology to *Drosophila spalt* and known to play an important role in development (Al-Baradie et al., 2002; Borozdin et al., 2004; Kohlhase et al., 1996). Mutations of *SALL4* in humans has been reported in the Okihiro/Duane-Radial Ray syndrome, an autosomal dominant disorder which is characterized by radial ray defects and is associated with defects in multiple organs (Al-Baradie et al., 2002). Recent studies have shown that SALL4 plays an essential role in pluripotent maintenance of murine ES cells; decreasing

SALL4 expression by siRNA resulted in the differentiation of ES cells to the trophoblast lineage (Zhang et al., 2006).

In view of the common roles of STAT3 and SALL4 in maintaining the pluirpotency of ES cells and their importance in oncogenesis, this chapter investigated if there is also a functional link between STAT3 and SALL4. In this chapter, I have shown that the SALL4 promoter carries four STAT3 binding consensus sequences. STAT3 indeed binds to these four sites and regulates the gene expression of *SALL4*, with the binding region starting at -199 being the most influential. This concept is in keeping with the observations that site-directed mutations at the all four STAT3-binding sites significantly reduced the luciferase activity in our *SALL4*/luciferase reporter gene assay, and the reduction was most dramatic with mutations introduced at the -199 site. Lastly, the functional relationship between STAT3 and SALL4 was confirmed using a native human cell line that expressed both STAT3 and SALL4.

For the first time, I demonstrated that aberrant activation of SALL4 was detectable in various breast cancer cell lines. SALL4 expression in these cells is likely to be functional since it is localized to the nucleus. Moreover, by comparing the results of MDA-MB-231 and MCF7, the nuclear SALL4 protein level appears to correlate with that of pSTAT3, although more comprehensive study is required to confirm this impression. The finding of relatively consistent expression of SALL4 in various breast cancer cell lines is initially surprising, considering that SALL4 is believed to be a stem cell marker (Elling et al., 2006; Zhang et al., 2006). Nevertheless, it is not uncommon for stem cell markers to be

expressed in carcinoma cells. For instance, Oct4, one of the key regulators of ES cells, was detectable in a variety of human cancers including that of breast, lung, pancreas, colon and ovary (Monk and Holding, 2001). In addition to Oct4, a relatively recent study by Ezeh *et al.* identified expression of *Nanog*, another regulator of ES cells, in breast tumors and cell lines but not in normal breast tissues (Ezeh et al., 2005). Although the biological role of Nanog and Oct4 in cancer is largely unknown, the expression of these two markers may correlate with tumor aggressiveness. Specifically, a recent study reported that expression of *Nanog* and *Oct4* is associated with a high-grade histology (Ben-Porath et al., 2008). Similar to *SALL4*, the interactions between STAT3 and both Oct4 and Nanog have been reported to be essential for the maintenance of murine ES cells in a pluripotent state (Chambers, 2004) and for oncogenesis (Gibbs et al., 2005). For instance, Oct4 is reported to provide anti-apoptotic effects in murine ES cells and this effect may be mediated by the STAT3 signaling pathway (Guo et al., 2008). Co-expression of STAT3, Oct4 and Nanog has been reported in bone sarcoma cells, although whether STAT3 is responsible for the expression of Oct4 and Nanog in these cells were not addressed (Gibbs et al., 2005). In light of the findings in this chapter, it is tempting to speculate that STAT3 may directly contribute to the aberrant expression of these stem cell markers in various forms of human cancer.

SALL4 appears to be biologically significant in the breast cancer cell lines, since siRNA-mediated downregulation of SALL4 expression in MDA-MB-231 led to a significant decrease in cell growth. Interestingly, it has been reported that breast cancer cells treated with Ad-STAT3DN also showed cell cycle arrest and it is possible that this biological

effect may be mediated via SALL4. While the focus of this chapter is related to the functional link between STAT3 and SALL4, our laboratory is currently further investigating the clinical and biological significance of SALL4 in breast cancer.

The oncogenic potential of SALL4 has been recently revealed by multiple studies. The *SALL4* transgenic mice exhibit myelodysplastic-like features including ineffective hematopoiesis and increased proportion of immature cells (Ma et al., 2006). The *SALL4* level was found to be consistently increased with progression to leukemia. SALL4 is known to directly target the *BMI-1* gene, a marker that is important in predicting the disease progression of acute myeloid leukemia (Yang et al., 2007). Interestingly, SALL4 have been reported to directly bind to β-catenin and activate the Wnt canonical pathway signaling pathway (Ma et al., 2006). Another study reported that the Wnt canonical signaling pathway can directly regulate SALL4 expression by direct interaction of T-cell factor/lymphoid enhancer factor 1 (TCF/LEF1), members of the Wnt signalling pathway, with the *SALL4* promoter (Bohm et al., 2006). This particular interaction has been elucidated in various vital physiological phenomena (Arias, 2001; Huelsken et al., 2001; Tan et al., 2005), such as maintenance of pluripotent cells in a proliferative state, promotion of self-renewal in stem cells (Reya et al., 2003; Willert et al., 2003) and regulation of cell proliferation, migration, apoptosis and differentiation (Miller, 2002). Taken together, it is highly likely that SALL4 cooperates with the Wnt canonical pathway, as well as the STAT3 signaling pathway, to promote tumorigenesis in various human malignancies.

To conclude, this chapter describes a novel link between STAT3 and SALL4 where *SALL4* is reported to be a direct target gene of STAT3. Furthermore, I delineated the specific STAT3-binding region required for optimal transcriptional activation of SALL4. This data suggests that STAT3 and SALL4 likely cooperate, in both physiological and pathologic states.

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Chapter 6 ♦

Discussion

6.1. Thesis overview

In this thesis, I have extensively evaluated the role of STAT3 in oncogenesis by identifying novel upstream regulators and direct target genes of STAT3. Previous studies demonstrated aberrant activation of STAT3 in a number of human cancer cell lines and tumors (Bowman et al., 2000; Bromberg, 2002; Garcia et al., 2001; Huang et al., 2000; Lin et al., 2000), including 90% of ALK^+ALCL cases and approximately 50% of primary breast cancer cases (Chiarle et al., 2005; Diaz et al., 2006; Dolled-Filhart et al., 2003; Khoury JD, 2003). However, STAT3 signaling is biologically complex and the exact mechanism by which STAT3 mediates oncogenesis is not fully understood. I hypothesize that aberrant activation of STAT3 signaling, through aberrant cytokine up-regulation and induction of downstream target genes, is clinically and biologically significant in the pathophysiology of a variety of human cancers. The three main objectives in this thesis is to increase our understanding of the effects of STAT3 in cancer by identifying specific targets of STAT3 that may contribute to the pathogenesis of cancer, by exploring the possible interactions of STAT3 with other oncogenes, and by identifying ESPs and corresponding receptors that may contribute to STAT3 activation in cancer. Through the identification of novel upstream and downstream targets of STAT3 and its biological effects, my work furthered our current understanding of the mechanism involved in the aberrant activation of STAT3 in oncogenesis. These novel findings may possibly lead to the development of novel anti-cancer treatments designed specifically to disrupt these targets of STAT3.

6.2. STAT3 activation by upstream regulators

One of the primary objectives in this thesis was to identify novel upstream regulators of STAT3 that may contribute to its oncogenic effects in cancer. The identification of upstream regulators of STAT3 is essential, not only to increase our understanding of how STAT3 becomes aberrantly activated in cancer, but also to provide us with possible therapeutic targets to treat STAT3-driven cancers.

As described in chapter 1, there are a number of different groups of cytokines that are reported to contribute to the aberrant activation of STAT3. Since many different cytokines are known to activate STAT3, it is not surprising that persistent activation of STAT3 can be a consequence of aberrant cytokine signaling that is either by autocrine or paracrine stimulation. One major cytokine that is well known to stimulate STAT3 activation is IL-6. In multiple myeloma cells, STAT3 activation is dependent on a well characterized IL-6 autocrine pathway, which contributes to the increase in survival observed in multiple myeloma (Catlett-Falcone R, 1999). In addition, autocrine IL-6-mediated STAT3 activation and VEGF up-regulation was also observed in lung adenocarcinoma (Lee et al., 2008). Another cytokine that have been implicated in STAT3 activation is IL-9. Similar to IL-21, IL-9 is part of the common γ-chain family of cytokine receptors and requires the heterodimeric receptor complex IL-9Rα and γ-chain to exert its function. Recently, autocrine release of IL-9 have been described in ALK^+ALCL and resulted in JAK3/STAT3 activation to contribute to the pathogenesis of this type of lymphoma (Qiu et al., 2006).

In light of these findings, it is likely that an autocrine cytokine stimulatory pathway is a mechanism commonly utilized by cancer cells to promote tumorigenesis. This allows the tumor cells to independently produce its own cytokine, which in turn will affect cell growth and survival. In this thesis, a preliminary study was done using an oligoarray to identify cytokines that are expressed in an ALK^+ALCL tumor. A number of cytokines were identified in the oligoarray but the only cytokines that were of interest were the ones that also had the corresponding receptor expressions, since cytokines are unable to exert their biological functions in the absence of their corresponding receptor complexes. Therefore, the two cytokines that I chose to focus on were IL-22 and IL-21. Both IL-22 and IL-21 have been reported to stimulate STAT3 signaling in other human cell types and cancer models (Nagalakshmi et al., 2004; Ueda et al., 2005), but the expression and effects of these two cytokines and their corresponding receptors have not been described in ALK^+ALCL. Further studies in this thesis reported the activation of STAT3 by both IL-22 and IL-21 autocrine stimulatory pathways in ALK^+ALCL (chapter 3 and 4). Of interest, Il-22R1 is normally absent in T-cells, making ALK^+ALCL a perfect model to study this aberrant stimulator of STAT3 signaling. Activation of STAT3 by IL-22 and IL-21 resulted in an increase in cell growth and tumorigenic potential in ALK^+ALCL cell lines. Moreover, a novel function for NPM-ALK was described in chapter 3 where the oncogenic fusion gene was found to be responsible for the aberrant expression of IL-22R1 in ALK^+ALCL.

6.3. STAT3-dependent activation of target genes

The second study objective of this thesis is to identify novel downstream targets of STAT3 in cancer. The identification of STAT3 target genes and its functions will assist us in understanding the effects of STAT3 signaling in cancer cells. Similarly to the first thesis objective, it may also provide us with possible therapeutic targets to treat STAT3-driven cancers.

As described in chapter 1, STAT3-regulated genes include ones that are involved in cell cycle progression, apoptosis, angiogenesis, cell invasion, metastasis, and immune function (Dauer et al., 2005; Galkin et al., 2007). TIMP1 have been suggested to be a direct target of STAT3 due to the presence of STAT3-binding sites on the *TIMP1* promoter (Bugno et al., 1995). A previous study by Lai *et al.* reported that STAT3 plays a direct role in up-regulating TIMP1 expression in ALK^+ALCL and that TIMP1 expression directly correlated with STAT3 activation in ALK^+ALCL (Lai et al., 2004). In chapter 2, I demonstrated that STAT3 directly up-regulates TIMP1 expression in breast cancer cell lines and tumors, a novel finding that is supported by the presence of STAT3-binding sites on the *TIMP1* promoter. This up-regulation of TIMP1 by STAT3 modulated the invasive potential of breast cancer cells lines. In addition, high STAT3 activation was found to inversely correlate with vascular and lymphatic invasion, a finding that is contradictory to the pro-oncogenic properties of STAT3 observed in other human tumors. However, it is well recognized that while some breast cancer patients present with metastatic diseases but relatively small primary tumors, other patients present as localized disease despite the presence of large primary tumors. Overall,

through up-regulation of many downstream targets, including TIMP1, STAT3 appears to carry an important role in modulating many biological aspects of breast cancer, including cell survival, proliferation, and invasive potential.

The inverse correlation observed between STAT3 and lymphatic/vascular invasion in chapter 2 is supported by a previous finding showing STAT3 nuclear localization in node-negative breast cancer was associated with significantly improved survival at both 5 and 20 years (Dolled-Filhart et al., 2003). In contrast, a latter study using immunohistochemical analysis reported high nuclear localization of pSTAT3 in stage III breast carcinoma (Diaz et al., 2006). Unlike the immunohistochemical study carried out in chapter 2, which included stages II, III, and IV breast carcinomas, Diaz and colleagues only compared pSTAT3 in stage III breast carcinoma with non-neoplastic breast tissue. The differences observed between these studies may also be partially due to the processing time of the tissue resection since phosphorylation STAT3 is subjected to tyrosine phosphatase activity. It is important to note that the primary breast tumors obtained for the study in chapter 2 were subjected to fixation in a timely fashion to avoid reduced STAT3 phosphorylation levels. Also, the primary focus of the latter study was to correlate STAT3 activation with chemotherapy resistance, not invasion (Diaz et al., 2006).

Overall, the metastatic process is complex and involves multiple steps, including invasion of tumor cells into tissue surrounding the primary tumor, entering the lymphatic vessels or the bloodstream, survival of circulatory cells, arrest in a new organ, extravasation into

the tissue, initiation and maintenance of growth, and vascularization of the metastatic tumor (Steeg P S, 1998). Chapter 2 suggests that differences in the STAT3 activity and TIMP1 expression in breast cancer may determine how early metastasis occurs during the course of the disease. However, aside from STAT3, numerous factors, including other oncogenes and tumor suppressors, are involved in this complex process and further work is essential to fully understand the complex network that is involved in controlling a cell's ability to stay localized in particular site or successfully metastasize to other regions. For example, experiments using SCID mice may be advantageous to directly assess the pathogenetic importance of STAT3 and TIMP1 in cancer. Injection of breast cancer cell lines permanently expressing TRE-STAT3C into mice, followed by tetracycline administration (through drinking water) at different dosage, will allow for sufficient evaluation of the oncogenic potential of STAT3 signaling at different levels of activation. Furthermore, cDNA microarray may be performed on breast cancer cell lines permanently expressing TRE-STAT3C to further detect novel molecular targets of STAT3.

In chapter 5, the direct association between STAT3 and SALL4 was first reported. SALL4 is a stem cell marker that is known to be important in the maintenance of embryonic stem cells in a pluripotent state (Zhang et al., 2006) and have recently been reported to be constitutively active in AML (Ma et al., 2006). This thesis reports that STAT3 will directly bind to the specific STAT3-binding sites on the *SALL4* promoter to regulate the transcriptional activation of SALL4. In addition, a specific functional region on the *SALL4* promoter was delineated and appears to be essential for SALL4 activation.

Interestingly, I demonstrated that SALL4 is also expressed in breast cancer cell lines. This finding can be considered controversial since SALL4 is believed to be a stem cell marker and is not expressed in normal breast tissues, however, stem cell markers have been previously reported to be expressed in cancer cells (Ezeh et al., 2005; Zhang et al., 2006). Preliminary data discussed in chapter 5 revealed that SALL4 may have growth promoting properties in breast cancer cells, however further studies are required to fully understand the biological effects of aberrant activation of SALL4 in breast cancer and other human cancers. The next stage of this research will be to further evaluate the biological relationship between STAT3 and SALL4 using ES cells. It is expected that shutdown of STAT3 activation in ES cells will also result in ablation of SALL4 activation. Also, further work is required to further demonstrate the link between STAT3 and SALL4 in cancer. Immunohistochemical analysis on primary breast tumor sections should be performed to determine whether there is a correlation between STAT3 and SALL4 expression. Also, it will be of interest to determine whether SALL4 is constitutively activated in other human cancers and whether this activation is primarily due to STAT3 activation or other co-activators. For instance, our lab has identified SALL4 expression in ALK^{+}ALCL, a type of lymphoma well known to have extremely high STAT3 activation. In contrast, SALL4 expression and STAT3 activation is absent in MCL.

6.4. Therapeutic implications for STAT3

The knowledge of signaling pathways has proven critical for the development of cancer therapies and efforts have been made to establish STAT3 as a valid molecular target for

therapeutic intervention (Al Zaid Siddiquee and Turkson, 2008; Bromberg J and Darnell, 2000; Turkson J, 2000). As previously described in chapter 1, there are several anti-cancer strategies that have been pursued to directly target STAT3. Although targeted drugs have achieved great success in the initial clinical stages, drug resistance due to mutations and genetic alterations is inevitable and elucidates the importance of multi-target drugs. The use of 'cocktails' or a combination of agents that target different characteristics of the tumor cells is a potential approach to avoid drug resistance and to achieve maximal therapeutic benefits (Galkin et al., 2007). This emphasizes the importance of this thesis in identifying novel targets of STAT3 that may potentially lead to the development of new anti-cancer drugs.

The work in this thesis provides a framework for alternative strategies of targeting the STAT3 signaling pathway in cancer. Chapter 3 suggests that development of receptor-ligand interaction antagonists to prevent IL-22-dependent activation of STAT3 may be a possible strategy. Treating cancer cells that express IL-22R1 with the cytokine antagonist IL-22BP or IL-22 neutralizing antibodies may inhibit STAT3 activation and prevent tumor cell growth. In ALK^+ALCL where NPM-ALK has been implicated in the aberrant expression of IL-22R1, current therapeutic approaches targeting NPM-ALK may possibly act as an indirect IL-22 antagonist as well.

Recombinant IL-21 is currently in clinical trials for treatment of patients with melanoma and renal carcinoma and is found to efficiently induce apoptosis of cancer cells with patients experiencing mild toxicity (Davis et al., 2007b; Thompson et al., 2008). In

addition, recombinant IL-21 in combination with rituximab in $CD20^+$ B-cell non-Hodgkin's lymphoma or with cetuximab in colorectal cancer enhanced antibody-dependent cellular cytotoxicity (Davis et al., 2007a). Chapter 4 reports an adverse role for IL-21 cytokine in ALK^+ALCL where cell growth promotion was observed, suggesting a dual function for IL-21 that is dependent on cell type. Therefore, an IL-21 neutralizing antibody treatment may possibly be explored for treatments of such cancers as ALK^+ALCL.

As previously mentioned, therapeutic approaches have also been made to inhibit known STAT3 target genes. The work in chapter 5 identified SALL4 as a novel target gene for STAT3 in cancer. Therefore, another possible therapeutic strategy may involve targeting SALL4 and its involvement in malignant progression. This strategy may be possible after further progress is made in understanding the mechanism involved in aberrant SALL4 activation in a variety of human cancers.

6.5. Closing remarks

Overall, this thesis has provided insight into the role of STAT3 signaling in the pathogenesis of cancer. The identification of novel upstream regulators and direct target genes of STAT3 have increased our current understanding of the STAT3 pathway and allowed us to further delineate the mechanism by which STAT3 mediates oncogenesis. The work in this thesis will provide a framework to further identify critical targets in the STAT3 pathway and its clinical relevance, to develop new diagnostic tests that will readily detect STAT3-driven cancers, and to ultimately develop novel therapeutic

strategies that target the inherent abnormalities of cancer cells. Identification of novel upstream stimulators of STAT3, downstream targets of STAT3, including the interaction between STAT3 and other oncogenes, may possibly lead to the development of novel anti-cancer treatments designed specifically to disrupt these targets of STAT3. Signal transduction plays a major role in cancer and the mechanisms by which these signaling pathways mediate carcinogenesis are being increasingly explored to identify novel molecular targets of therapeutic importance. This thesis has furthered our understanding of STAT3, one of the major signal transduction pathways implicated in cancer.

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